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Histomorphological evaluation of stromal collagen in canine mammary tumours: A study using picrosirius red stain and polarizing microscopy#

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Abstract

Mammary gland tumours are considered as the most common neoplasms in sexually intact female dogs. Incidence of mammary tumours, especially the malignant ones are found to be rare in dogs younger than five years of age. Latest reports indicate that more than 75 per cent of canine mammary tumours (CMTs) are of malignant type. Recent research has demonstrated that in breast cancer, apart from changes in the parenchyma, major modifications also occur in the stroma or tumour microenvironment, which surrounds the neoplastic cells and these changes are now understood to be crucial factors in the onset and progression of tumour. The tumour microenvironment consists of multiple cell types such as fibroblasts, leukocytes, adipocytes, myoepithelial cells, endothelial cells and the extra cellular matrix (ECM). Numerous studies have indicated that the primary protein in the ECM, collagen, has the potential to give physical, biochemical, and biomechanical impulses to both tumour and non-tumour cells, thus regulating the growth and spread of cancer. The current study employed special staining using picrosirius red to demonstrate the stromal collagen in CMTs and utilised polarised microscopy to analyse the birefringence of collagen fibres in tumours. Picrosirius red staining for evaluation of alterations in stromal collagen fibres was identified to be highly efficient in determining the prognosis of CMTs.

Keywords: *Collagen, canine mammary tumour, picrosirius red, polarised microscopy*

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Mammary gland tumours are the most frequent neoplasms in sexually intact female dogs in veterinary practice, similar to breast cancer which is the most common malignancy in women. As dogs age, their risk of developing mammary tumours rises and becomes considerable around the age of seven or eight. The prognosis is directly correlated with a number of variables, including tumour size, lymph node involvement, occurrence of distant metastases, angiogenesis, histological type and tumour grade. The interaction between the tumour cells and the associated stroma has found to influence the growth and spread of cancer and has led to widespread speculation that the tumour microenvironment has an impact in the process of tumourigenesis. The "seed and soil" hypothesis put forwarded by Paget (1889) postulated that for the optimal growth of the tumour cells (seeds), a favourable host microenvironment (soil) is essential. Hence, these stromal changes are now recognised as key attributes in the initiation and progression of breast cancer as well as prospective therapeutic targets.

The ECM is a complex network of proteins that surrounds and stabilises cells which mainly consists of three types of proteins: structural proteins (e.g., collagen/ elastin), glycoproteins (e.g., fibronectin) and proteoglycans (e.g., chondroitin sulfate). The primary protein in the ECM, collagen, has the ability to give physical, biochemical, and biomechanical impulses to both tumour and non-tumour stromal cells, thereby regulating the initiation and progression of various tumours (Conklin *et al*., 2011). Collagen undergoes structural changes and realignment during cancer, and thus collagen density and fibre organisation influence the progression of human breast cancers (HBCs). Individual collagen fibres within the tumour are straight, whereas they are wavy in more distant normal tissue and are of intermediate straightness close to the tumour. Collagen realignment fosters the migration of epithelial tumour cells during the invasion process (Walker *et al*., 2018). In HBCs, increase in breast density, identified as the earliest sign of malignancy, has been found to be associated with increased fibrillar collagen deposition (Provenzano *et al*., 2008). However, the studies related to fibroplasia and role of collagen in CMTs are very much limited and hence, the present study was undertaken with the objective of demonstrating the alterations occurring in stromal collagen, by employing picrosirius red as a differential stain.

Materials and methods

Twenty-five CMT suspected cases presented to the University Veterinary hospitals at Mannuthy and Kokkalai formed the material for the present study. Clinical history of the affected animals, gross features of the tumour mass such as size, shape, colour and location were recorded at the time of tissue collection. After surgical excision, biopsy samples were collected. Samples were placed in 10 per cent neutral buffered formalin immediately following the surgery and further processing was done after 48-72 hours of fixation. The paraffin embedded tissues were sectioned at five micrometre thickness and was stained using Haematoxylin and Eosin (Suvarna *et al.,* 2018). The sections were examined under the light microscope and the various histologic subtypes were determined according to the standard classification system put forwarded by Goldschmidt *et al*. (2011). Histological malignancy grading (HMG) was done as described by Clemente *et al.* (2010) which is a modification of Elston and Ellis (1991) method of grading HBCs. The extent of tubule formation, degree of nuclear pleomorphism and the number of mitoses per 10 high power fields were used as the criteria to grade the tumours. Further, the stroma associated with tumours was classified into different types depending on the extent of fibroplasia. The stroma having high and moderate degrees of fibroplasia were stained with picrosirius red to study the alterations in the distribution of collagen in various histo-types and malignancy grades of CMTs. The tissue samples obtained from normal mammary gland of a female dog carcass brought for necropsy at the Department of Veterinary Pathology, CVAS, Mannuthy served as control for the study.

Picrosirius red staining

Paraffin sections were cleared in xylene and dehydrated. Nuclear staining was

done using Weigert's iron haematoxylin solution for 10 minutes followed by washing in running tap water for another 10 minutes. Sections were stained in picrosirius red (solution prepared by dissolving 0.1 g Sirius red F3B in100 mL of saturated aqueous solution of picric acid) for one hour and washed in acidified water which was prepared by adding 1.5 mL glacial acetic acid to 200 mL distilled water. Most of the water from slides was removed by air drying. It was followed by dehydration in three changes of 100 per cent alcohol and then cleared in two changes of xylene and mounted in DPX (Luna, 1968). Upon staining, collagen appeared red in colour under light microscope and based on the amount of collagen deposited; the cancer associated stroma was further classified into three *viz*. collagen rich stroma, stroma with intermediate amounts of collagen and collagen poor stroma.

Polarised microscopy

The formalin fixed paraffin embedded tissue samples at a thickness of 5 µm was stained with picrosirius red. The sections were analysed using a Leica DM 2700P microscope (Wetzlar, Germany) equipped with filters to provide linearly polarised illumination. Tissue images were obtained with 20X and 50X objective lens and recorded by a digital camera (Leica Digital FireWire Camera (DFC).

Results and discussion

The present study was based on the data procured from twenty-five dogs suspected for mammary tumours and out of the 25-tumour suspected mammary gland excision biopsies, all were neoplastic and 24 (96 per cent) belonged to malignant type whereas one case (four per cent) was a benign tumour. Similar findings were reported by Mathew *et al.* (2019), Christy *et al.* (2022) and Devi (2022) that majority of the CMT samples obtained from dogs of Thrissur district belonged to malignant category. No CMT cases in dogs below 4 years and above 14 years of age were observed during the course of the study and the mean age of occurrence in the studied population was 9.04±0.54 years. This is in line with the findings of Sharma *et al.* (2018) who reported 9.1±0.64 years as the mean age of incidence of CMTs. Caudal abdominal glands were observed to be affected more frequently, which accounted for sixty per cent of the total population studied. Patel *et al.* (2019) also reported that the highest incidence of CMTs was noticed in caudal abdominal glands. The malignant CMTs in the present study were histologically graded as per Clemente *et al.* (2010). Among the 24 malignant tumours studied, only one was a Grade I or well differentiated tumour (four per cent), fourteen (58.3 per cent) were Grade II, moderately differentiated tumours and nine (37.5 per cent) were Grade III or poorly differentiated tumours. Malignant CMTs were further divided into a number of subtypes according to their histological characteristics, the majority of which were simple carcinomas and according to the current study, ductal carcinoma, which made up 33.3 per cent of all CMTs, was the most prevalent histological type among the other subtypes. Other histological subtypes identified were tubulopapillary, solid, cribriform, anaplastic, spindle cell and squamous cell carcinomas of mammary gland. Two cases of

Table 1. Histological classification of malignant tumours (n=24)

SL. No	Histological type	No. of cases
1	Tubulopapillary carcinoma	4
2	Cribriform carcinoma	1
3	Squamous cell carcinoma	1
4	Ductal carcinoma	8
5	Solid carcinoma	6
6	Carcinosarcoma	2
7	Spindle cell carcinoma	1
8	Anaplastic carcinoma	1

carcinosarcoma were also identified.

Stromal assessment revealed that, out of the 25 CMT samples studied, 10 had high or severe fibroplasia where the cancer associated stroma was highly rich in collagen, 12 samples had moderate fibroplasia with intermediate amounts of collagen deposition in the stroma and three samples had mild fibroplasia with low amounts of collagen deposition (Fig. 1 and 2).

To demonstrate the stromal fibroplasia, a special staining technique using picrosirius red was employed in those cases having high or moderate amounts of stromal collagen. Junqueira *et al*. (1979) used picrosirius red staining (PSR) to develop a simple and sensitive method for identifying fibrillar collagen networks in tissue sections. According to Devi *et al.* (2022), PSR staining was highly effective in demonstrating various collagen signatures associated with CMTs. Collagen was easily detected with PSR in the current study as it imparted a red colour under bright field microscopy. Varying degrees of collagen deposition could be identified with PSR staining which ranged from mild, through moderate to high (Fig 3-5). The extent of collagen deposition in relation to various histo-types of CMTs was evaluated and the results are given in Table 2.

The results indicated that the benign variant namely lipoma had only low amount of stromal collagen, while the aggressive histo-types of CMTs had either moderate or rich collagenous stroma with an exception of anaplastic carcinoma. Similarly, the collagen deposition in various malignancy grades of tumours was also analysed and the results are shown in Table 3.

It was identified that CMT samples with moderate to high fibroplasia either belonged to Grade II or Grade III tumours indicating that enhanced collagen deposition in cancer-associated stroma is a diagnostic signature of high-grade mammary tumours.

The progression of changes in collagen fibres was recorded in picrosirius red stained sections under polarised microscopy by observing the variations in birefringence. Collagen in normal mammary gland depicted

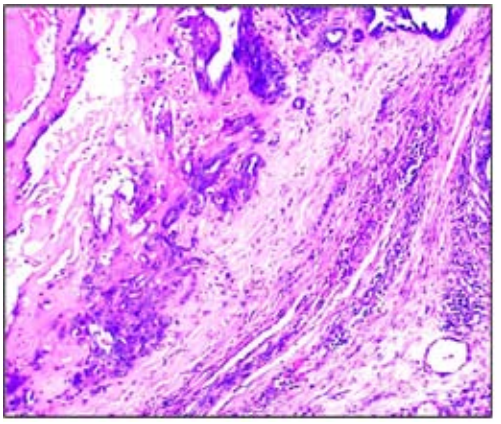

Fig.1. Stromal assessment- High stroma (H & E x 200)

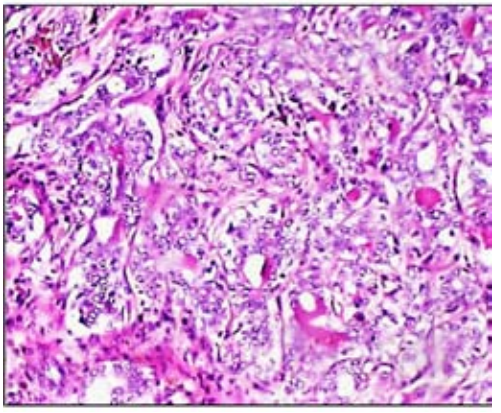

Fig. 2. Stromal assessment. Low stroma (H & E x 200)

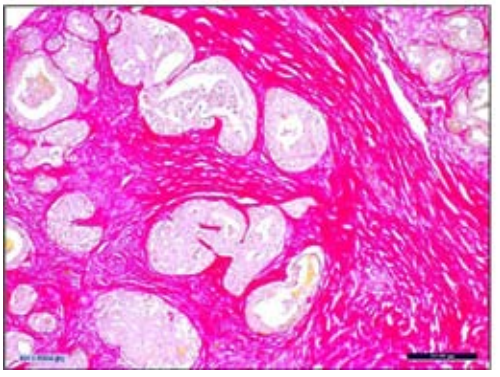

Fig. 3. High amounts of collagen in the stroma (PSR x 200)

a reddish orange birefringence whereas in moderately differentiated tumours, yellowish orange birefringence could be appreciated (Fig.6 and 7). In poorly differentiated tumour, yellowish green birefringence was observed (Fig.8). In normal mammary glands, dense, tightly packed and highly aligned collagen

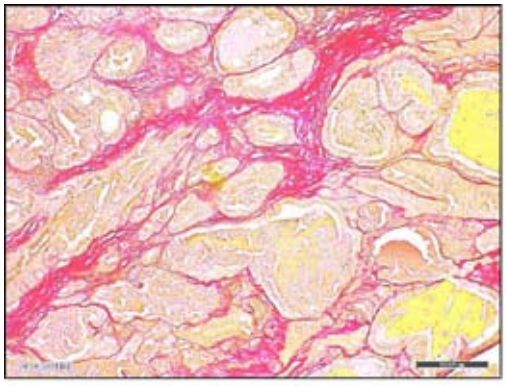

Fig. 4. Moderate amounts of collagen in the stroma (PSR x 200)

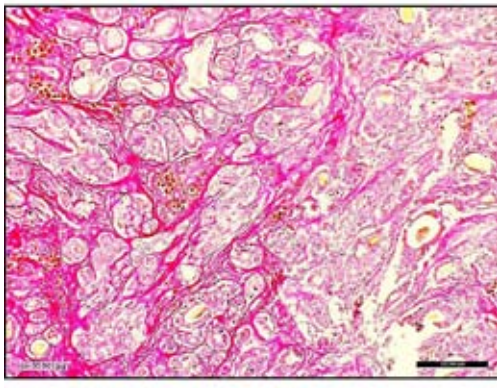

Fig. 5. Low amounts of collagen in the stroma (PSR x 200)

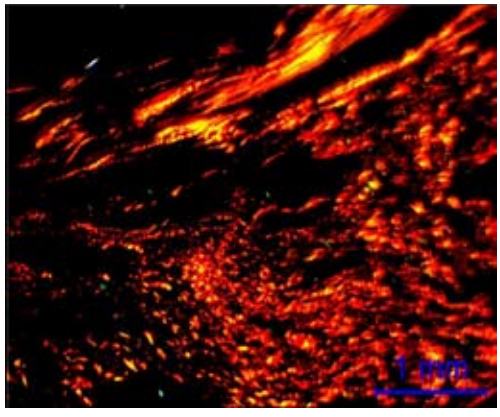

Fig. 6. Collagen of normal mammary gland stroma-Reddish orange birefringence (PSR x 200)

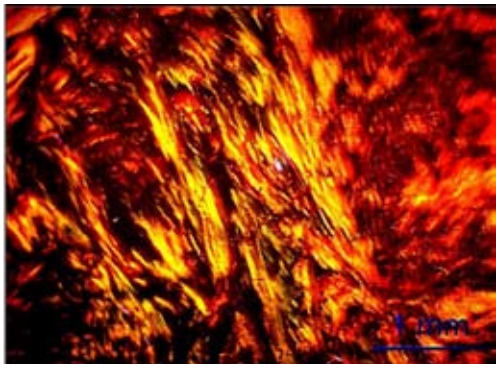

Fig.7. Yellow green birefringence – moderately differentiated tumours (PSR x 100)

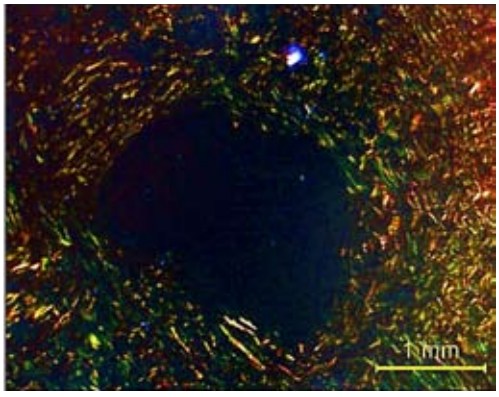

Fig. 8. Greenish yellow birefringence in poorly differentiated tumour (PSR x 100)

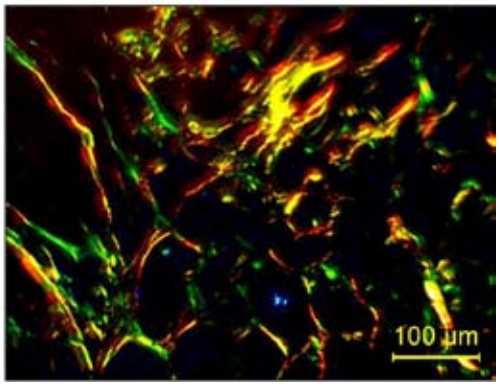

Fig. 9. Very loose thin myxoid matrix of poorly differentiated tumour with collagen fibres giving yellowish green birefringence (PSR x 100)

was observed. However, as the tumour progressed from moderately differentiated to poorly differentiated ones, the collagen fibres were observed to be packed in a less dense, haphazard and loose manner. The sections of high-grade tumours showed an increase in greenish hue as compared to the yellowish orange hue in low grade tumours (Fig. 9).

The observations were similar to the findings of Dayan *et al.* (1989) that tightly packed and better aligned collagen fibres showed polarisation colours of longer wavelengths. Similarly, as per Peddapelli *et al*. (2019), thin,

Table 2. Classification of stroma in different histological types of CMTs (n=25)

Histological type	Collagen poor stroma	Stroma with intermediate amounts of collagen	Collagen rich stroma
Tubulopapillary carcinoma		2	2
Cribriform carcinoma		1	
Squamous cell carcinoma		1	
Ductal carcinoma		5	3
Solid carcinoma		3	3
Carcinosarcoma			2
Spindle cell carcinoma	1		
Anaplastic carcinoma	1		
Lipoma	1		
Total	**3**	**12**	**10**

Table 3. Classification of the stroma in different grades of CMTs (n=25)

Tumour grades	Stroma with low amounts of collagen	Stroma with intermediate amounts of collagen	Stroma with high amounts of collagen
Benign (Lipoma)	1		
Grade I		1	
Grade II		9	5
Grade III	2	2	5
Total	**3**	**12**	**10**

parallel, and loosely arranged greenish-yellow collagen fibres were associated with high recurrence rate and biological aggressiveness of odontogenic tumours. In the current study, similar findings could be appreciated where the polarised colours of PSR stained sections reflected the density of packing and alignment of collagen fibres with the hue component of the image ranging from red, orange, yellow, and green as the tumour progressed.

Conclusion

The findings of the current study revealed that in addition to the neoplastic epithelial cells, the cancer associated stroma also harbours many indicators reflecting the malignancy status of CMTs, of which the ECM collagen is the most important one. The extent of collagen deposition as well as the packing and alignment could be used to predict the prognosis and design treatment in CMTs. The present study has also indicated that polarised microscopy of picrosirius red stained tumour sections can be of great use in studying the collagen realignment occurring in CMTs.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Christy, M.J., Sajitha, I.S., Devi, S.S., Dhanush, KB. and Sudheesh, S. N. 2022. Study on histopathological subtypes and grading of canine mammary tumours. *J. Vet. Anim. Sci*. **53**: 85-88.

Clemente, M., Perez-Alenza, M.D., Illera, J.C. and Pena, L. 2010. Histological, immunohistological, and ultrastructural description of vasculogenic mimicry in canine mammary cancer. *Vet. Pathol*. **47**: 265-274.

Conklin, M.W., Eickhoff, J.C., Riching, K.M., Pehlke, C.A., Eliceiri, K.W., Provenzano, P.P., Friedl, A. and Keely, P.J. 2011. Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am. J. Clin. Pathol*. **178:** 1221-1232.

Dayan, D., Hiss, Y., Hirshberg A., Bubis, J.J., Wolman, M. 1989. Are the polarization colors of picrosirius red-stained collagen determined only by the diameter of the fibers? *Histochemistry*. **93**(1): 27-29.

Devi, S.S. 2022. Histomorphological, immunohistochemical and molecular studies on desmoplastic reactions in canine mammary tumours. *Ph.D. thesis,* Kerala Veterinary and Animal Sciences University, Pookode, 148p.

Devi, S.S., George, A.J., Dhanushkrishna, B., Prasanna, K.S., Radhika, G., and John Martin, D. 2022. Desmoplastic reactions associated with malignant canine mammary tumours. *J. Vet. Anim. Sci*. **53**(4): 694-698.

Elston, C.W. and Ellis, I.O. 1991. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*. **19**: 403-410.

Goldschmidt, M., Pena, L., Rasotto, R. and Zappulli, V. 2011.Classification and grading of canine mammary tumours. *Vet. Pathol*. **48**: 117-131.

Junqueira, L.C.U, Bignolas, G. and Brentani, R.R. 1979. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem. J*. **11:** 447-455.

Luna, G. 1968. *Manual of Histological Staining Methods of the Armed Forces Institute of Pathology*. (3rd Ed.). Mc Graw Hill Book Company, New York, 207p.

Mathew, R., Sajitha, I.S., Nair, S.S., Krishna, B.D. and Abraham, M.J. 2019. Canine mammary tumours: Histological malignancy grading as a prognostic indicator. *Pharma Innovation*. **8**: 149-151.

Paget, S., 1889. The distribution of secondary growths in cancer of the breast. *The Lancet. **133***: 571-573.

Patel, M.P., Ghodasara, D.J., Raval, S.H. and Joshi, B.P. 2019. Incidence, gross morphology, histopathology and immunohistochemistry of canine mammary tumors. *Ind. J. Vet. Sci. Biotech*. **14**: 40-44.

Peddapelli, K., Rao, V., Kumar, M.P., Sravya, T. and Rakesh, D. 2019. Analysis of collagen fibers in keratocystic odontogenic tumor and ameloblastoma: A polarizing microscopic study. *Ind. J. Dent. Res*. **30**: 731-735

Provenzano, P.P., Inman, D.R., Eliceiri, K.W., Knittel, J.G., Yan, L., Rueden, C.T., White, J.G. and Keely, P.J. 2008. Collagen density promotes mammary tumor initiation and progression. *BMC Med.* **6**: 1-15.

Sharma, N., Gupta, A., Bhat, R. and Shah, O. 2018. Epidemiological Studies on Canine Tumours in Jammu. *Int. J. Livestock Res*. **8**: 246-254.

Suvarna, K., Layton, C. and Bancroft, J.D. 2018*. Bancroft's Theory and Practice of Histological Techniques.* (8th Ed.). Elsevier Health Sciences, Churchill, 584p.

Walker, C., Mojares, E., del Río Hernández, A. 2018. Role of extracellular matrix in development and cancer progression. *Int. J. Mol. Sci*. **19:** 3028.

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