STROMAL FIBROPLASIA AS A PROGNOSTIC INDICATOR IN CANINE MAMMARY TUMOURS

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ABSTRACT

Mammary tumours are considered as the most important neoplasms in dogs especially in sexually intact female dogs. Prognosis is directly associated with various factors such as tumour size, metastases, involvement of lymph node, tumour type and grade. Recently, tumour microenvironment (TME) has gained huge importance and proved to be a key regulator of breast cancer's biologic behaviour, according to mounting data. The capacity of extracellular matrix components like collagen to alter their intrinsic structure and alignment aids in tumour invasion and metastasis. We present a case study of 25 canine mammary tumours (CMTs), where the stroma associated with the malignancy was specifically considered while histomorphologically analysing the tumours. The majority of the tumours were malignant (96 per cent) and one was identified to be benign. Simple carcinomas made up the majority of tumours, followed by mixed types. Cancer associated stroma was assessed by routine H and E staining and additionally by performing immunohistochemistry technique with anti-cytokeratin antibody to access the relative abundance of neoplastic epithelial cells. Special stains such as Picrosirius red, Masson's trichrome and Herovici were employed to demonstrate the desmoplastic stromal reaction and varying grades of collagen could be observed in the stroma of different tumours. The extent of fibroplasia was found to be directly proportional to the grade and size of tumour. Thus, results of the present study indicated that the cancer associated stroma significantly influence the CMT progression and clinical outcome.

Keywords: Canine mammary tumours, collagen, fibroplasia, stroma

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INTRODUCTION

Mammary gland tumours are the most frequent neoplasms in sexually intact female dogs and are common in veterinary practice (Case *et al*., 2017). In dogs under the age of five, mammary tumours, especially malignant forms, are incredibly rare. As dogs become older, the risk of developing tumours rises and becomes considerable by the time they become seven or eight years old (Withrow *et al*., 2013). Histopathology is the gold standard diagnostic method to classify and provide prognostic information on dogs with mammary tumours. The classification of canine mammary tumours (CMTs) has great clinical importance and is based on the subtype and degree of malignancy (Borghesi *et al.,* 2021). Approximately 50 per cent of canine mammary gland tumours are malignant, and the prognosis is directly associated with factors such as the tumour size, lymph node involvement, blood vessel growth, histological type and grade. The dynamic mutual cross talk between the neoplastic cells and surrounding stroma drives the process of tumour genesis in many cases including mammary tumours of dogs (Reis *et al*., 2017; Amini *et al*., 2020). Mammographic density study in human breast cancer has revealed the direct link of increased stromal collagen with tumour progression and metastasis. Increased breast density is not only associated with enhanced epithelial and stromal cellularity but also with increased fibrillar collagen deposition (Provenzano *et al*., 2008). The studies on cancer associated stroma and stromal collagen in CMTs are very much limited except for some recent studies which indicated alteration in the structure and organisation of collagen fibres in these tumours (Kovalenko *et al.,* 2021; Devi *et al.,* 2022). Hence, the present study was formulated with the objective of histomorphologically analysing the prognostic significance of stroma associated with CMTs with special reference to fibroplasia and collagen deposition.

MATERIALS AND METHODS

The present study comprised a total of 25 CMT cases that were presented to the University Veterinary hospitals at Mannuthy and Kokkalai. The tumour samples were fixed in 10 per cent neutral buffered formalin for histopathological studies. At the time of tissue collection, the clinical history of the affected animals by the tumour as well as the gross characteristics of the tumour mass, such as size, shape, colour and location were noted. Normal paraffin embedding procedures were used to process tumour tissue samples that had been fixed in 10 per cent neutral buffered formalin. Using a rotary microtome, serial sections were cut at a thickness of 5 microns, and the routine haematoxylin and eosin process was used to stain the sections. The sections were examined under the light microscope and classified to different histologic sub-types as described 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) grading method of human breast cancer. Scoring system is described as follows:

Tubule formation

Tubule formation in the section was assessed semi quantitatively and a score of one point was given when more than 75 per cent of the area was composed of definite tubules. Two and three points were given respectively when 10-75 per cent and < 10 per cent of the area was covered by tubules.

Nuclear pleomorphism

When the nuclei were small with minimum variation in size and had uniform chromatin, a score of one point was given. Nuclei larger in size with moderate anisokaryosis were given 2 points. When nuclei were vesicular and varying considerably in size and shape and bearing prominent nucleoli, 3 points were given.

Mitotic counts

Mitotic activity was assessed at a magnification of 400x (high power field, HPF), which provides a field area of 0.237 sq.mm. A minimum of 10 fields were examined. Upto nine mitoses per 10 HPF were given one point, 10-19 mitotic figures per 10 HPF were scored two points and 20 or more mitotic figures per 10 HPF were given three points.

After scoring in each of the above aspects, the scores were added to get a number between three and nine. Then the histological malignancy grade (HMG) was allocated as described below:

HMG I (low grade) - three to five points, well differentiated

HMG II (intermediate grade) - six or seven points, moderately differentiated

HMG III (high grade) - eight or nine points, poorly differentiated

Tumour stromal ratio (TSR) was assessed as per the criteria described by Xu *et al.* (2020) in invasive breast cancers. The histological sections were examined under 10x objective lens and a 50 per cent cut-off point was selected to divide patients into high TSR/ low stroma, moderate TSR/ moderate stroma and low TSR/ high stroma groups. Additionally, immunohistochemistry (IHC) using anti-cytokeratin antibodies was performed on the formalin fixed paraffin embedded (FFPE) tissues as per technique elaborated by Ramos-Vara, (2005).

The TSR was determined by assessing relative abundance of positively stained neoplastic epithelial cells in each microscopic field.

Further, the tumours having high or moderate stroma were analysed for the extent of fibroplasia using H and E stained sections and employing various special staining techniques like Masson's trichrome, Picrosirius red (PSR), and Herovici.

RESULTS AND DISCUSSION

Twenty five cases of CMT suspected growths were considered for the study. The lowest and highest ages of CMT occurrence in the animals under study were 4 and 14 years, respectively. The mean age of occurrence of CMTs in the studied population was 9.04±0.54

years. The finding was in agreement with that of Sharma *et al*. (2018) where in the mean age for CMT occurrence was reported as 9.1±0.64 years. Female dogs were found to have the highest incidence of CMT, while male dogs were less affected. The most affected glands in the present study were caudal abdominal (60 per cent), followed by the inguinal glands (32 per cent). The majority of the tumours were noticed as soft to hard masses that ranged in colour from greyish to yellowish white. Out of the 25 CMT suspected samples, 24 were malignant tumours which accounted for 96 per cent and one was found to be benign and a similar observation was reported by Kavya *et al.* (2020) where 92.5 per cent of the CMTs were identified as malignant ones.

Based on the histological features, malignant CMTs were classified into different subtypes and most of which were simple carcinomas (Table 1). Filho *et al*. (2010) observed that the most frequent histologic type of mammary tumour was simple carcinoma. The current study revealed that ductal carcinoma was the most common histological type among other subtypes, accounting for 33.3 per cent of all CMTs (Fig 1). This is consistent with the findings of Mathew *et al.* (2019), Christy *et al.* (2022) and Devi *et al.* (2022) who stated that ductal carcinomas were more common when compared to other histological types among dogs of Thrissur district. Tubulopapillary carcinoma showed a tubular and papillary patterns of epithelial cell proliferation with delicate fibrovascular stalks (Fig 2) as observed by Gamba *et al*. (2011). Solid carcinoma consisted of sheets of cells without lumina as described by Misdorp *et al*. (1999) (Fig 3). Squamous cell carcinoma of mammary gland was similar to those that occur in the skin, where islands and cords of epithelial cells were noticed with the formation of keratin pearls. In spindle cell carcinoma, the neoplastic cells were predominantly spindle shaped. Majority of the cells and nuclei were large and fusiform with moderate pleomorphism. Anaplastic carcinoma had neoplastic cells that were often individualized or grouped in small nests; they were also round, oval, or polygonal, with moderate to abundant eosinophilic cytoplasm. Multiple variably sized nucleoli were frequently observed. These findings are similar to those described by Goldschmidt *et al.* (2011).

The malignant tumours obtained in the current study were histologically graded according to Clemente *et al.* (2010). Among the 24 malignant tumours studied, only one belonged to grade I (4 per cent), fifteen (63 per cent) were grade II and eight (33 per cent) were grade III (Fig 4). In the present study, all the four tubulopapillary carcinomas and cribriform carcinoma belonged to grade II. Majority of the ductal carcinomas obtained in the study were of grade II. Out of the six solid carcinomas, four of them were Grade III and two were grade II. The results are in agreement with the observations of Rezaie *et al.* (2009) that simple carcinomas were more aggressive and represented either grade II or III. Carcinosarcomas obtained in the present study were of grade III and the findings were

Table 1. Histological classification of 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

in accordance with the results of Lin *et al*. (2020) that carcinosarcomas belonged to higher grades.

Tumour to stromal ratio (TSR) was reported as a valuable prognostic indicator in CMTs (Devi *et al*., 2022) and hence, the TSR was assessed using routine H and E stained histological sections and by performing IHC for cytokeratin. In the present study, 40 per cent samples had low TSR/ high stroma, 48 per cent had moderate TSR/ moderate stroma and 12 per cent had high TSR/ low stroma (Fig 5 and Fig 6). The stroma rich tumours obtained in the present study were typically of high grades and none of them were grade I. De Kruijf *et al.* (2011) reported that compared to patients with stroma-poor breast cancers, those with stroma-rich cancers had a greater risk of relapse. The present study also identified that increase in stromal proportion corresponded to increased malignancy and hence, evaluation of TSR in presurgical biopsies could help in easy prediction of prognosis and clinical outcome in CMTs.

Canine mammary tumours with high and moderate stroma (n=22) were considered for further analysis and based on the extent of fibroplasia, stroma was divided into three types *i.e.* stroma with severe fibroplasia, stroma with moderate fibroplasia and stroma with mild fibroplasia. Out of the 22 CMT cases, 50 per cent had stroma with moderate fibroplasia whereas 41 per cent had severe fibroplasia. Mild fibroplasia was observed only in nine per cent of tumours. Severe fibroplasia was observed as a feature in high grade tumours like carcinosarcomas (grade III), solid carcinomas (grade III) and tubulopapillary carcinomas (grade II). This finding was in agreement with the studies by Shadan *et al*. (2017) who reported that increased desmoplasia was associated with aggressive human breast cancers (HBCs). Thus, it was evident from the present study that increased desmoplasia was a feature of high grade CMTs.

In the present study, collagen could be easily detected with PSR as it provided a red colour to collagen under bright field

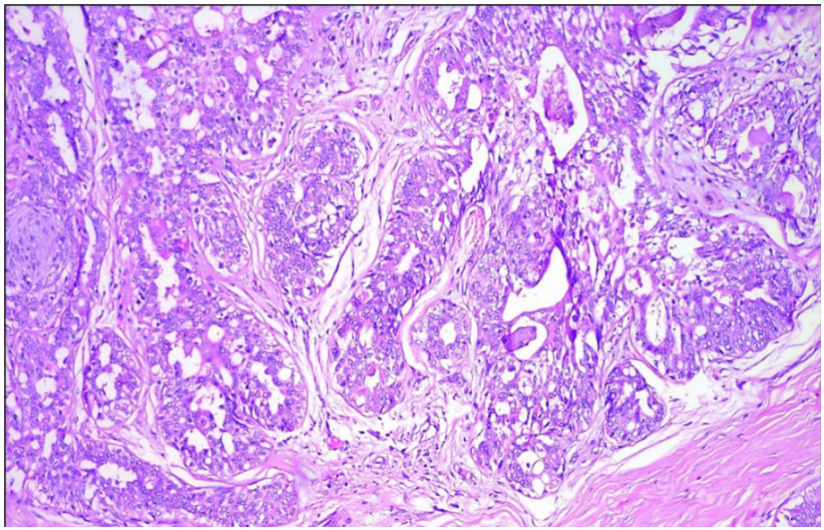

Fig 1. Ductal carcinoma - cords of pleomorphic neoplastic cells surrounding slit-like lumina lined by multiple layers of cells (H&E x 200)

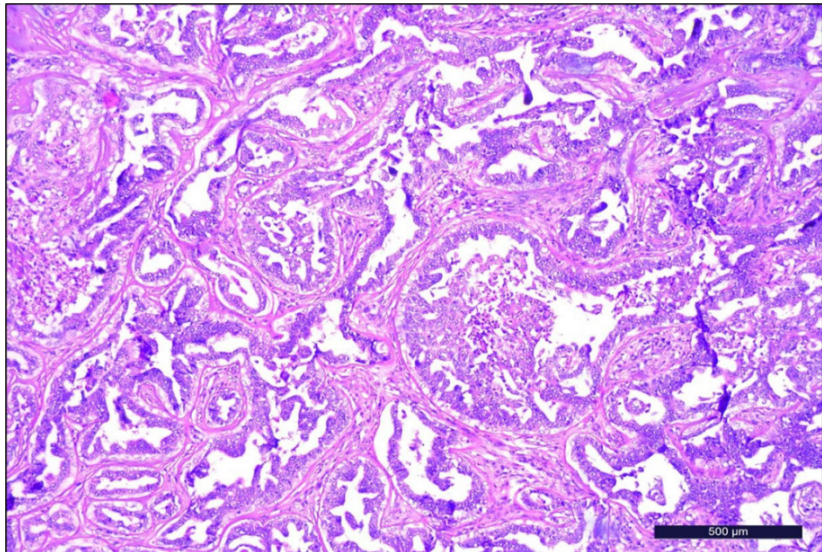

Fig 2. Tubulopapillary carcinoma- tubular and papillary pattern of epithelial cells with delicate fibrovascular stalks (H&E x 200)

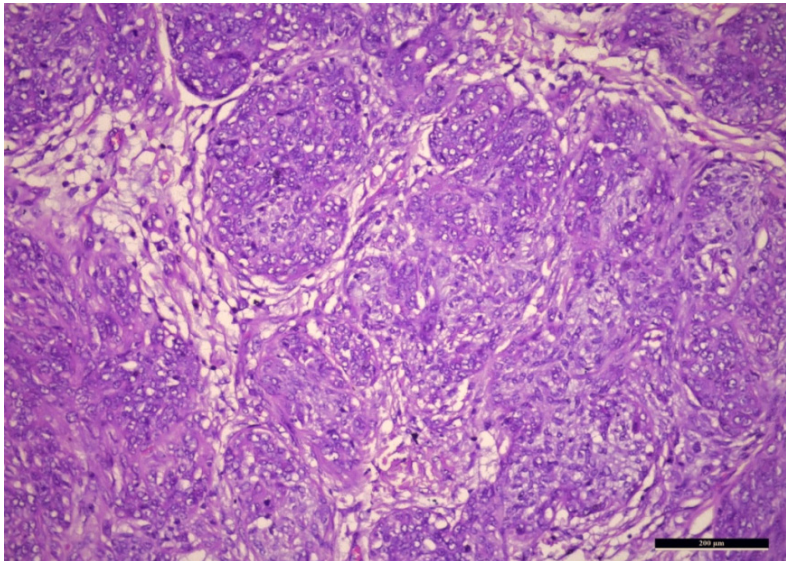

Fig 3. Solid carcinoma- sheets of cells without lumina (H&E x 200)

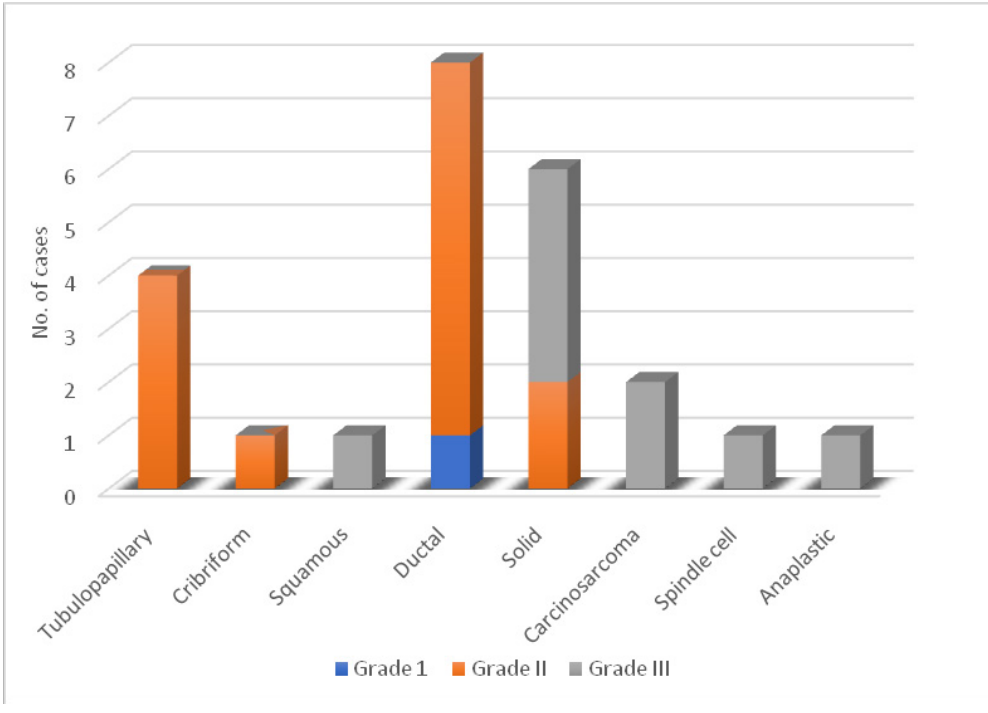

Fig 4. Different grades of tumour according to the histological subtypes of CMTs

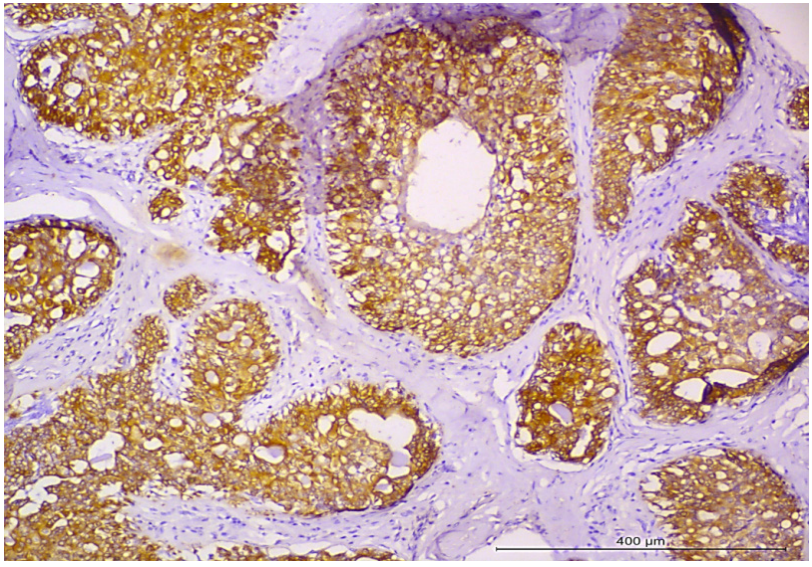

Fig 5. IHC for cytokeratin- Tumour with high TSR (low stroma) (IHC x 200)

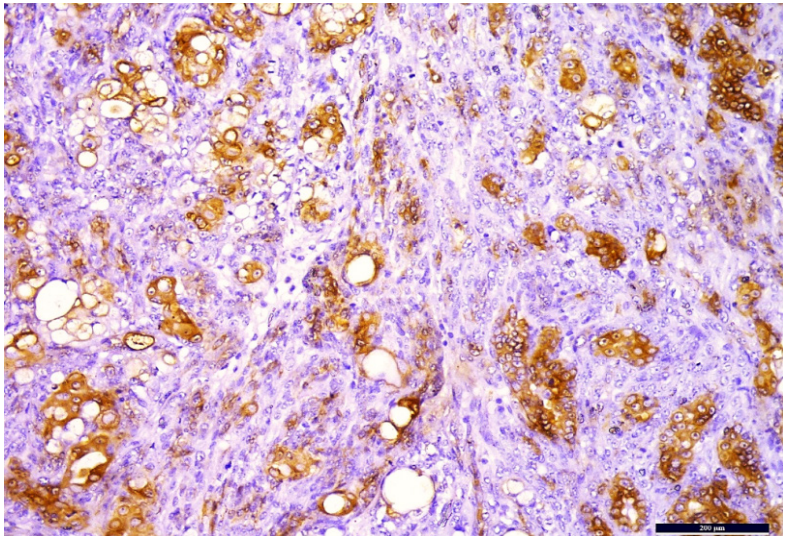

Fig 6. IHC for cytokeratin- Tumour with low TSR (high stroma)x 200

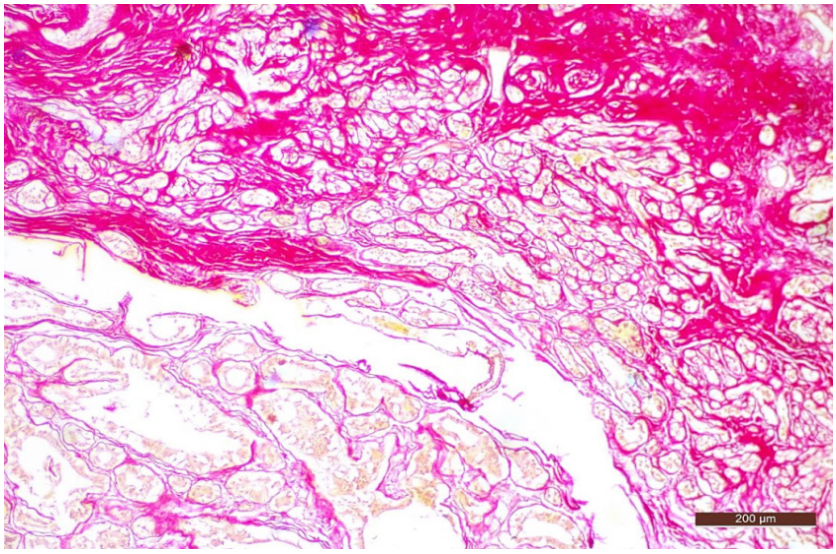

Fig 7. Picrosirius red staining 200x- Collagen appeared red in colour under bright field microscopy

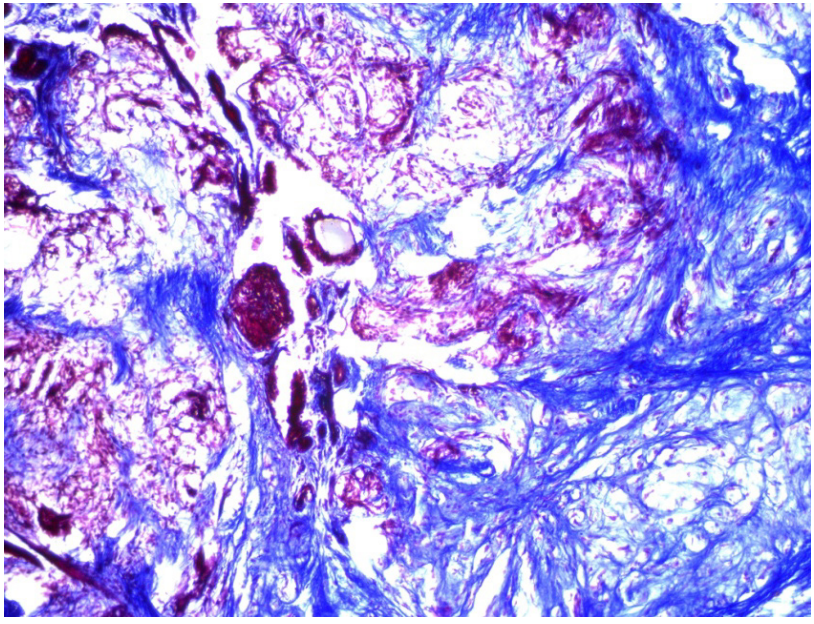

Fig 8. Masson's trichrome staining 200x- collagen appeared blue under bright field microscopy

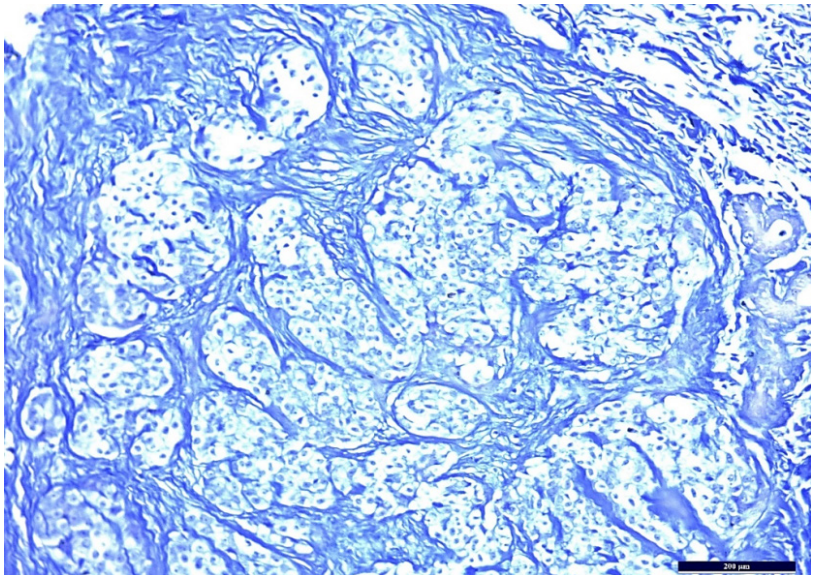

Fig 9. Herovici staining 200x- immature collagen appeared blue in colour indicating neocollagenisation in CMTs

microscopy (Fig 7). The present study identified varying degrees of collagen deposition with PSR staining which ranged from mild through moderate to high. According to Devi *et al*. (2022), PSR staining was highly effective in demonstration of various collagen signatures associated with CMTs.

Collagen fibres were stained blue with Masson's trichrome stain (Fig 8) and the present study could appreciate varying amounts of collagen deposition in different types and grades of tumours as observed in a study conducted by Borghesi *et al.* (2021). Immature collagen fibres could be observed as blue colour with Herovici staining (Fig 9). The presence of immature collagen indicated neo-collagenisation in mammary tumours and this observation was in agreement with the report of Luperallo (2013) who reviewed that the stroma of cancerous breasts underwent a desmoplastic reaction, which was defined by the excessive deposition of thick fibrous tissue mostly composed of newly generated collagen and other extra cellular matrix (ECM) components.

The present study revealed that along with neoplastic epithelial cells, the associated stroma had also got several clues indicative of the malignancy status of CMTs. The quantity as well as quality of stroma and extent of stromal fibroplasia could aid in the prediction of prognosis and clinical outcome in CMTs.

REFERENCES

Amini, P., Nassiri, S., Malbon, A. and Markkanen, E. (2020). Differential stromal reprogramming in benign and malignant naturally occurring canine

mammary tumours identifies disease-modulating stromal components. *Scientific Reports*, **10:** 1-13.

Borghesi, J., da Silva, M.G.K.C., Guimarães, K.D.O.P., Mario, L.C., da Anunciacao, A.R.D.A., Rabelo, A.C.S., Hayashi, R.G., Lima, M.F., Miglino, M.A., Favaron, P.O. and Carreira, A.C.O. (2021). Evaluation of immunohistopathological profile of tubular and solid canine mammary carcinomas. *Research in Veterinary Science*, **136**: 119-126.

Case, A., Brisson, B.K., Durham, A.C., Rosen, S., Monslow, J., Buza, E., Salah, P., Gillem, J., Ruthel, G., Veluvolu, S. and Kristiansen, V. (2017). Identification of prognostic collagen signatures and potential therapeutic stromal targets in canine mammary gland carcinoma. *PloS One,* **12**: 0180448.

Christy, M.J., Sajitha, I.S., Devi, S.S., Dhanush, K.B. and Sudheesh, S.N. (2022). Study on histopathological subtypes and grading of canine mammary tumours. *Journal of Veterinary and Animal Sciences,* **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. *Veterinary Pathology,* **47**: 265-274.

De Kruijf, E.M., van Nes, J.G., van de Velde, C.J., Putter, H., Smit, V.T., Liefers, G.J., Kuppen, P.J., Tollenaar, R.A. and Mesker, W.E. (2011). Tumour–stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. *Breast Cancer Research and Treatment*, **125**: 687-696.

Devi, S.S., George, A.J., Dhanushkrishna, B., Prasanna, K.S., Radhika, G. and John Martin, D. (2022). Histomorphological stratification of stromal types associated with canine mammary tumours. *Journal of Veterinary and Animal Sciences,* **53**: 688-693.

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.

Filho, J.C.O., Kommers, G.D., Masuda, E.K., Marques, B.M., Fighera, R.A., Irigoyen, L.F. and Barros, C.S. (2010). Retrospective study of 1647 mammary tumours in dogs. *Pesquisa Veterinaria Brasileira,* **30**: 177-185.

Gamba, C.O., Damasceno, K.A., Ferreira, E., Malta, M.C.C. and Cassali, G.D. (2011). Tubulopapillary carcinoma of the mammary gland in a maned wolf (*Chrysocyon*

brachyurus): histopathological and immunophenotypical analysis. *Arquivo Brasileiro de Medicina Veterináriae Zootecnia,* **63**: 1377-1381.

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

Kavya, M., Sajitha, I.S., Anoopraj, R., Anoop, S. and George, A. J. (2020). Occurrence and histopathological classification of canine mammary tumours in Wayanad, Kerala. *Pharma Innovation,* **9**(1): 220-223.

Kovalenko, M.S., Bilyi, D.D., Skliarov, P.M., Maslikov, S.N., Suslova, N.I., Spitsyna, T. L. and Yevtushenko, I.D. (2021). Prognostic markers of canine mammary tumours: Retrospective study of 142 cases. *Regulatory Mechanisms in Biosystems*, **12**: 649–654.

Lin, S., Liu, C., Tao, Z., Zhang, J. and Hu, X. (2020). Clinicopathological characteristics and survival outcomes in breast carcinosarcoma: A SEER population-based study. *The Breast*, **49**: 157-164.

Luparello, C. (2013). Aspects of collagen changes in breast cancer. *Journal of Carcinogenesis and Mutagenesis*, **13**: 7.

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**(4): 149-151.

Misdorp, W. (1999). Histological classification of the mammary tumors of the dog and the cat. World Health Organization International histological classification of tumors of domestic animals second series, **7:** 1-59.

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 Medicine*, **6:** 1-15.

Ramos-Vara, J.A. (2005). Technical aspects of immunohistochemistry. *Veterinary Pathology,* **42**: 405-426.

Reis, S., Gazinska, P., Hipwell, J.H., Mertzanidou, T., Naidoo, K., Williams, N., Pinder, S. and Hawkes, D.J. (2017). Automated classification of breast cancer stroma maturity from histological images. IEEE Transactions on *Biomedical Engineering,* **64**: 2344-2352.

Rezaie, A., Tavasoli, A., Bahonar, A. and Mehrazma, M. (2009). Grading in canine mammary gland carcinoma. *Journal of Biological Sciences,* **9**: 333-338.

Shadan, M., Khan, N., Khan, M.A., Ansari, H. and Zaheer, S. (2017). Histological categorization of stromal desmoplasia in breast cancer and its diagnostic and prognostic utility. *SSRG International Journal of Medical Science*, **4**: 8-11.

Sharma, N., Gupta, A., Bhat, R. and Shah, O. (2018). Epidemiological studies on canine tumours in Jammu. *International Journal of Livestock and Research,* **8**: 246-254.

Withrow, S.J., Vail, D.M. and Page, R. (2013). Withrow and Mac Ewen's small animal clinical oncology-E-Book. *Elsevier Health Sciences.*

Xu, Q., Yuan, J.P., Chen, Y.Y., Zhang, H.Y., Wang, L.W. and Xiong, B. (2020). Prognostic significance of the tumor-stromal ratio in invasive breast cancer and a proposal of a new Ts-TNM staging system. *Journal of Oncology*, 2020.