Final Report

UGC REPORT FINAL

1.	UGC Reference N. & Date	F.No. 41-117/2012(SR) Dated 11-07-2012
2.	Name of the Principal Investigator	Dr. Naresh Kumar Sood
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4.	Department and University/College where the project has undertaken.	Department of Veterinary Pathology, COVS, GADVASU, Ludhiana-141004.
5.	Title of the Project	'Studies on lymphangiogenesis in canine model of human breast cancer.' (UGC 12).
6.	Date of Implementation	1-07-2012
7.	Tenure of the project	3 years from1-07-2012 to 30-06-2015. Extended upto 31-12-2015
8.	Grants Received.	1st Installment- Rs 5,95,000/- 2nd Installment- 3,40,000 (Received in June 2015) Total- 9,35,000/-
9.	Objectives of the Project	To study extent of intra-tumoral and peri-tumoral lymphangiogenesis in canine mammary tumour. To study the role of lymphangiogenesis in invasion and metastasis of canine mammary tumour. To determine the molecular and signalling pathways involved in lymphoangiogenesis in canine mammary tumours as potential targets for research and anticancer therapy.

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10.	**Methodology**	1) All the dogs reporting to the Small animal Clinics of the department of Teaching Veterinary Clinical Complex were screened for mammary gland tumours and tissues and blood samples were collected for further processing. 2) Histopathology of suspected tumour tissue was done to confirm and classify the mammary tumours as per latest WHO classification. 3) Tumour grading and extent of presence of lymphatics / lymphangiogenesis and angiogenesis was determined by semi-quantitative analysis. 4) Immunohistochemical profiling of mammary gland tumours was done to study their morphological features and prognostic and predictive factors. 5) The degree of lymphangiogenesis/angiogenesis in tissue sections, as from above, was determined by immuno-reactivity for specific markers of lymphatic vessels i.e VEGFR-3, prox1, podoplanin , LYVE-1, COX2,NR2F2 and Claudin 5. 6) Quantify growth factors mediating lymphangiogenesis i.e VEGF-C, VEGF-D was done by Real Time PCR. 7) The prospective and archived samples of canine mammary tumours available in the department were subjected to additional diagnostic and prognostic markers

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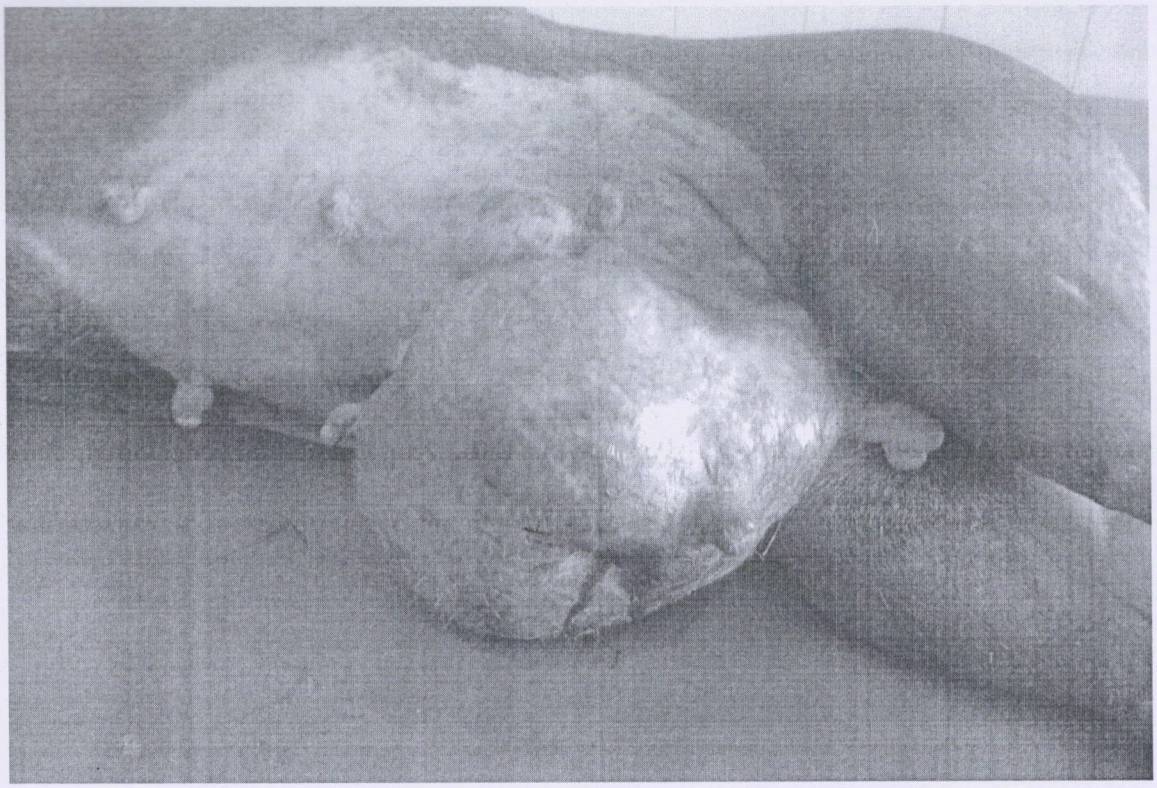
Progress made and salient research findings

Breast cancer over the last two decades has attracted worldwide attention due to high mortality rates and its heterogeneity. Mammary gland tumour is most common in canines especially in the un-spayed female dog. Studies on mammary gland tumour in canines have proved it to be an excellent model for human breast cancer . Clinical and pathological studies suggest that for carcinomas, the most common route of tumor cell metastasis is via lymphatics. Growth of new lymphatic vessels in the vicinity of solid tumors correlates with lymphatic metastasis, and various factors and receptors and signaling pathways have been identified which act as drivers of lymphangiogenesis.These factors, receptors and signaling pathways may therefore act as promising target for inhibition of lymphangiogenesis and hence for restricting metastasis.

Under the project the following studies were conducted w.e. f 2012- 2015

1. **Immuno-pathological and molecular studies on lymphangiogenesis in canine mammary tumour**

The study was conducted on tissue samples of 45 cases of canine mammary tumour (CMT).



CMT involving fourth pair of mammary gland

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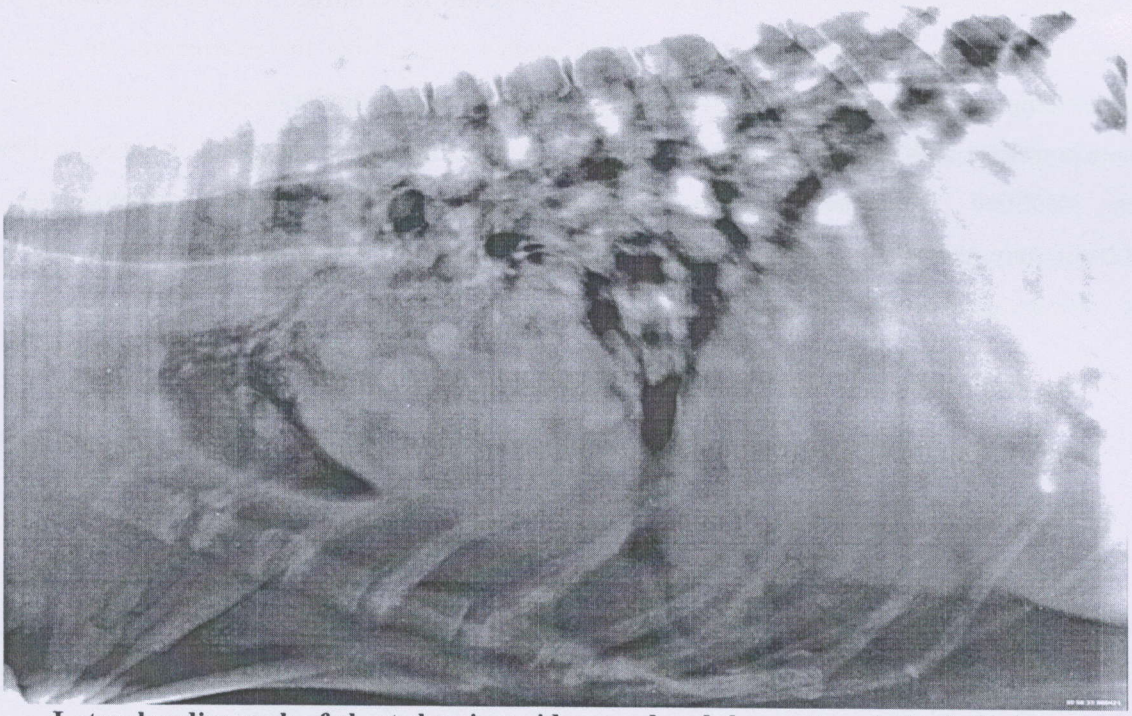

Lateral radiograph of chest showing widespread nodular pattern in both the lungs

Histopathology

On the basis of histopathology, 40 cases out of 45 showed significant intratumoral and peritumoral lymphatics and therefore were selected for further immunohistochemical staining. These cases depending upon histological features were split up into carcinoma (n=22), carcinosarcoma (n=13) and sarcoma (n=5) subtypes as per latest WHO classification of CMT.

Immunohistochemistry

a) Assessment of immunohistochemical staining and scoring- IRS

The IRS was calculated for each marker. Out of 40 cases of CMT, immunoreactivity for podoplanin was seen in all the 40 cases, VEGFR-3 in 39, LYVE-1 in 28 and PROX 1 in 25 cases, respectively. Podoplanin IRS was high in 31 cases and low in 9 cases. For LYVE-1, IRS was high in 13 and low in 15. Whereas, PROX 1 and VEGFR-3 showed high reactivity in 4, 33 and low in 21, 6 cases, respectively. A comparison of different histological subtypes revealed that the carcinosarcoma had high IRS for all the markers as compared to other two subtypes (Table 3).

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Podoplanin expression was limited to the cytoplasm of neoplastic cells and an increased expression of podoplanin was recorded in cases showing epithelial-mesenchymal transition (EMT) and at the invasive front. LYVE-1, PROX 1 and VEGF-D were also expressed in the cytoplasm of tumour cells , whereas, variable cytoplasmic as well as nuclear staining was observed for VEGFR-3. In addition, to the expression in tumour cells, expression for various markers (except VEGF-D) was also observed in tumour emboli. Furthermore, LYVE-1 staining was also seen in macrophages, lymphoid and plasma cells, besides tumour cells and their emboli. VEGF-D expression was also seen in myoepithelial cells besides, endothelial cells of some vessels. No immunoreaction for VEGF-C was, however, detected. Maan Whitney *U*-test revealed a significant difference in the expression level of PROX 1 in carcinosarcoma as compared to sarcoma (P=0.049).

b) Assessment of vessel counts

Using various markers, the lymphatics were detected both in intratumoral and peritumoral regions but their morphology ,density and immunoreaction was variable in the former and the latter as determined by a specific marker. In general, the lymphatics in intratumoral areas were small, thin, numerous and at times not clearly discernible, when compared with the peritumoral lymphatic vessels, which were larger, fewer and more conspicuous. I-LVD was observed highest with VEGFR-3 (35.94 ± 3.45) and podoplanin (31.95 ± 2.77), followed by LYVE-1 (11.11 ± 2.20) and PROX 1 (7.62 ± 1.11), whereas, P-LVD was recorded as 11.48 ± 1.32, 7.69 ± 0.51, 5.19 ± 0.96 and 3.48 ± 0.48 for these respective markers. VEGF-D expression did not show significant correlation with IRS, I-LVD or P-LVD of any of lymphatic markers (Podoplanin, LYVE-1, VEGFR-3), except with the IRS of PROX 1 (P = 0.010) in case of carcinosarcoma.

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Comparison of clinicopathological features with the I-LVD and P-LVD of various markers

Various clinicopathological features viz. age of the animal, size and grade of the tumour, LN metastasis, presence/absence of inflammation in the tumour, significant evidence of EMT and immunoreactivity for VEGF-D were compared and it was found that I-LVD had a significant correlation with the increase in size (P=0.017) of the primary tumour and the presence of EMT (P=0.015) as revealed by podoplanin expression. In addition, grade of the tumour (P=0.031) was significantly associated with P-LVD as determined by podoplanin staining. Moreover, I-LVD was significantly correlated with LN metastasis (P=0.041) as was elucidated by LYVE-1 immunoreaction. However, no significant correlation was detected between clinicopathological parameters and rest of the markers.

Survival Analysis

In our study, on basis of podoplanin reaction, in intratumoral area, the mean survival (months), with and without censoring was 14.56 and 13.50, when the lymphatic vessel density was high, and 13.92 and 12.72 months, when the lymphatic vessel density was low, respectively. In comparison, in peritumoral area, with and without censoring, the mean survival was 10.10 and 9.50 months, when lymphatic vessel density was high, whereas, it was 18.02 and 16.72 months when lymphatic vessel density was low, respectively. When the survival analysis was carried for PROX 1, in intratumoral area with low and high lymphatic vessel densities, the mean survival period was 15.75 and 12.70 months with censoring, and 14.37 and 11.85 months without censoring, respectively. In contrast, in peritumoral areas with censoring and without censoring, the mean survival period was 13.46 and 12.56 months, when lymphatic vessel density was high, 14.75 and 13.61 months when lymphatic vessel density was low, respectively.

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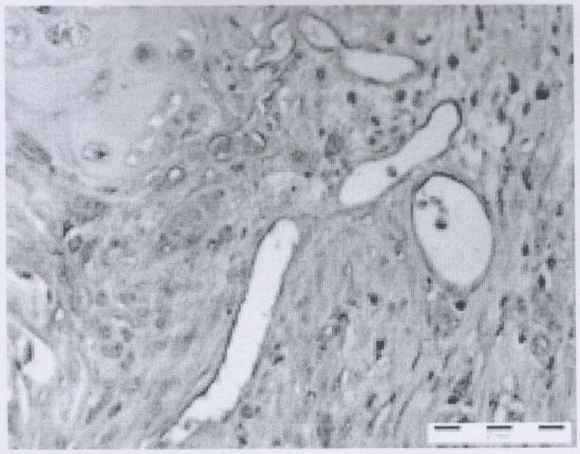
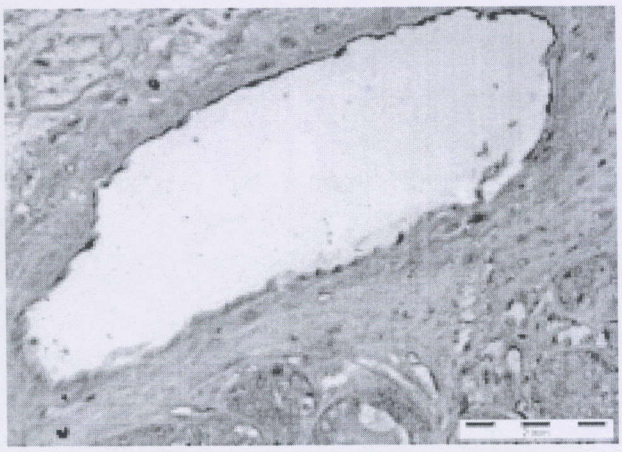
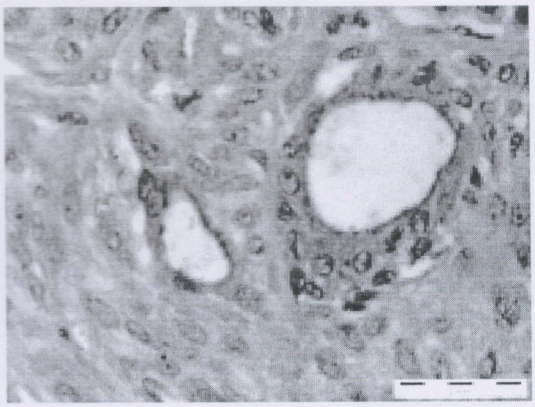


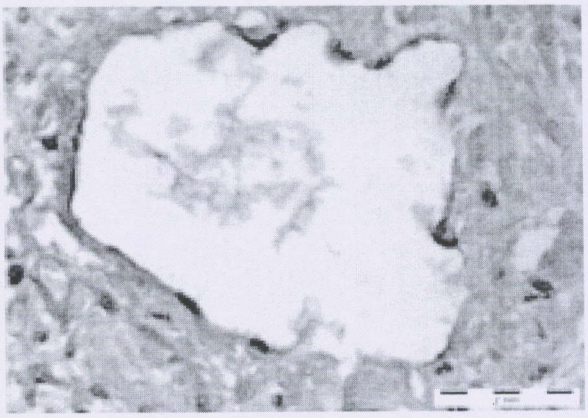
Podoplanin staining showing intratumoral lymphatic vessels



Peritumoral lymphatics stained with podoplanin



Intratumoral lymphatic vessels as stained with LYVE-1



Lymphatic endothelial cells in peritumoral areas stained with PROX 1.

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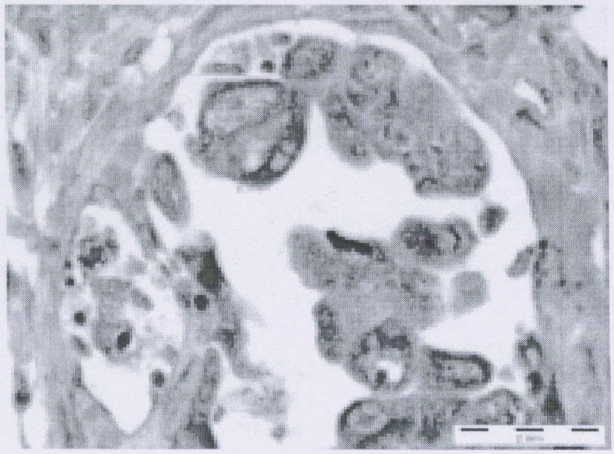
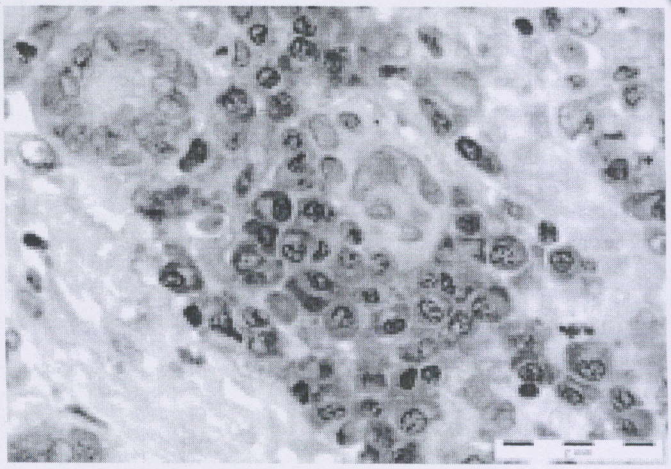
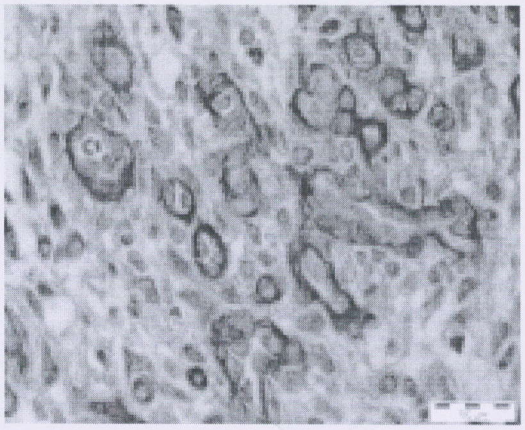


Tumour emboli in peritumoral lymphatic vessel besides a few fibroblasts, as stained with LYVE-1



Macrophages, lymphoid and more conspicuously plasma cells, stained with LYVE-1



Laminin immuno-stained blood vessels

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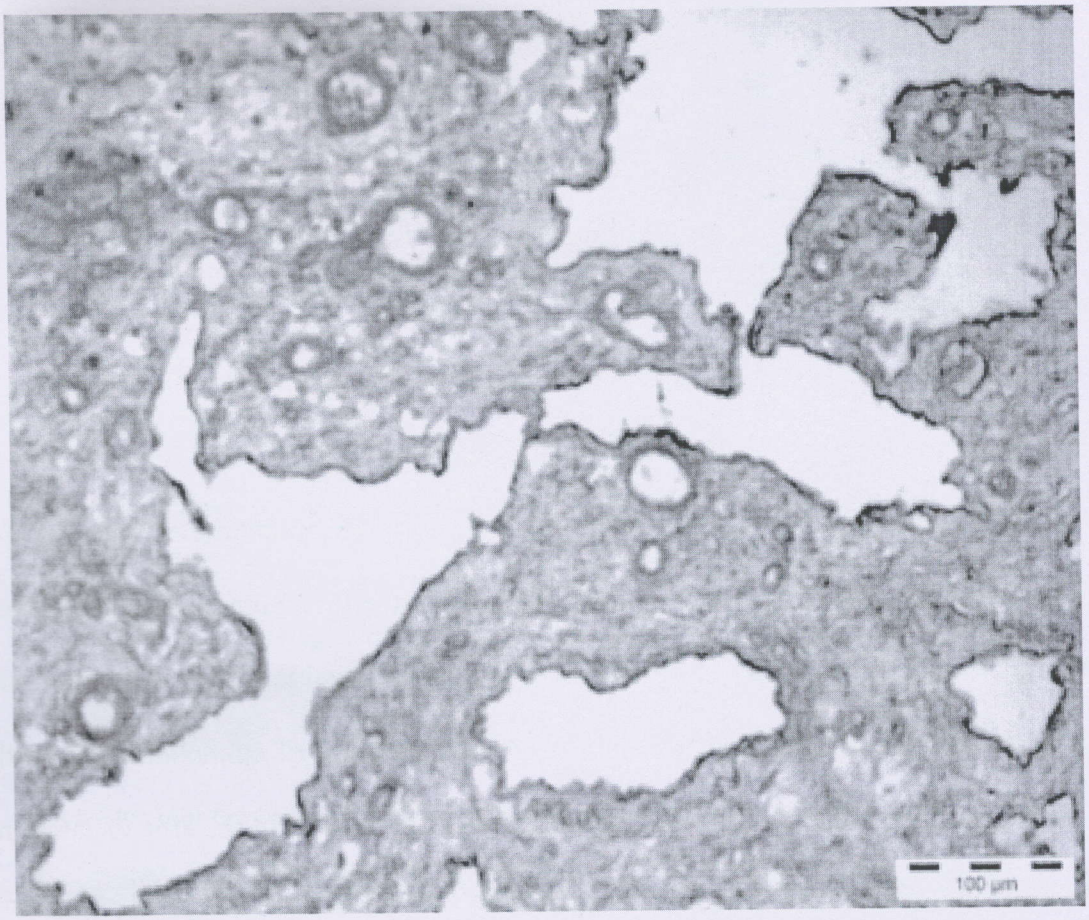

Double immunolabelling laminin stained blood vessels (brown), LYVE-1 stained lymphatics (grey)

2. Extended studies on novel markers of lymphangiogenesis and angiogenesis in canine mammary tumour

The second study was conducted on archived tissue samples from 53 cases of CMT available in the department. On the basis of WHO classification of CMT, 26 cases were classified as carcinomas (49%), 20 carcinosarcomas (37.8%) and 7 sarcomas (13.2%). Among carcinoma, 6 cases were further classified as simple carcinoma (23%), 17 as complex carcinoma (65.4%), 2 as squamous cell carcinoma (7.7%) and 1 case was of malignant myoepithelioma (3.8%). All the 20 cases of carcinosarcoma consisted of varying degree of epithelial and connective components. Among sarcomas, 5 cases were of osteosarcoma (71.4%) and 1 case each of hemangiosarcoma and fibrosarcoma (14.3%) respectively.

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Cyclooxygenase-2 (COX-2) has recently been considered to promote lymphangiogenesis by up-regulating vascular endothelial growth factor-C (VEGF-C) in breast cancer and little work has been conducted on this marker in CMT.

Immunoreactivity of COX-2 was observed in 52/53 (98%) cases of CMT. The reactivity of COX-2 was predominantly cytoplasmic and granular and it was detected in epithelial, myoepithelial, immature connective tissue and in EMT. The immunoreactivity of COX-2 was weak in epithelial cells as compared to the reactivity in the immature connective tissue, whereas, no immunoreactivity of COX-2 was detected in mature connective tissue. In addition, the positive reactivity of COX-2 was also detected in the perinuclear areas of epithelial cells, on the luminal side of the acini, endothelium of blood vessels, tumor emboli and in the area of inflammation. On the basis of staining intensity, 22 cases revealed weak immunoreactivity, 17 cases moderate and 13 cases revealed strong immunoreactivity, whereas, no immunoreactivity of COX-2 was detected in one case. The average immunohistochemical score of COX-2 in grade I tumours was 4.1, in grade II tumours 5.12 and in grade III tumours it was 5.92. A low positive, but non-significant correlation was observed between COX-2 expression and the grade of the CMT. More expression of COX-2 was associated with higher histologic grade of CMT, which suggested that COX-2 may play an important role in tumour differentiation and it may be associated with poor prognosis of CMT.

The work was continued on novel markers of lymphangiogenesis NR2F2 whih showed encouraging results. In addition, the angiogenetic marke claudin 5 was found to be expressed in cases of canine mammary hemangiosarcomas.

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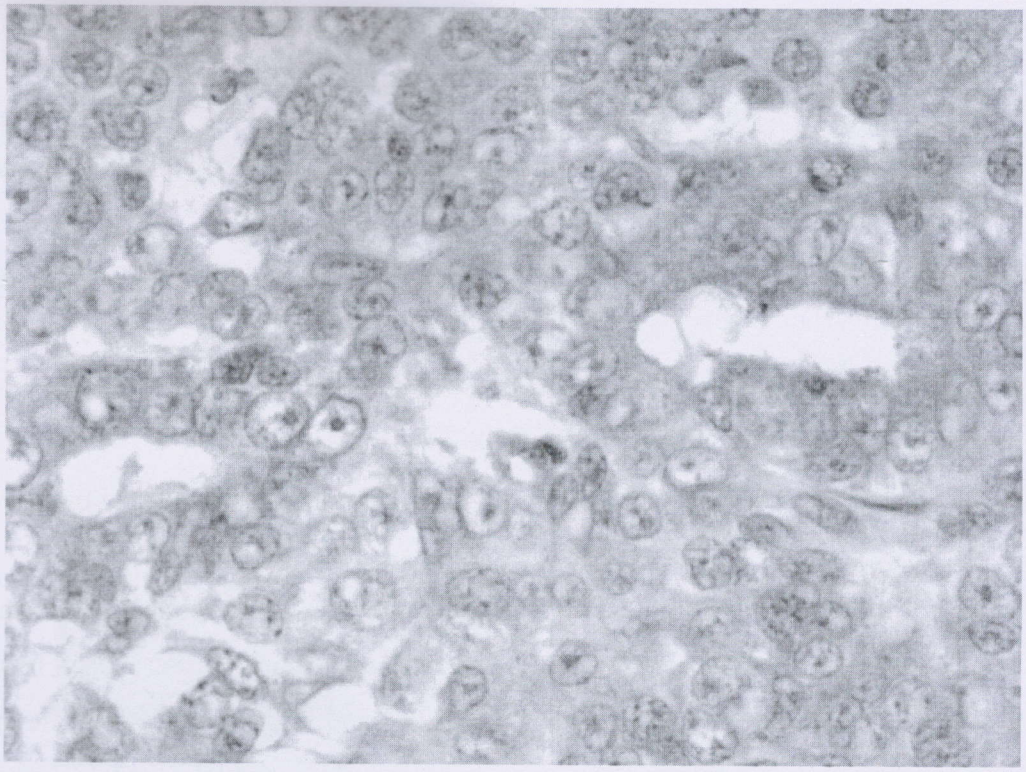
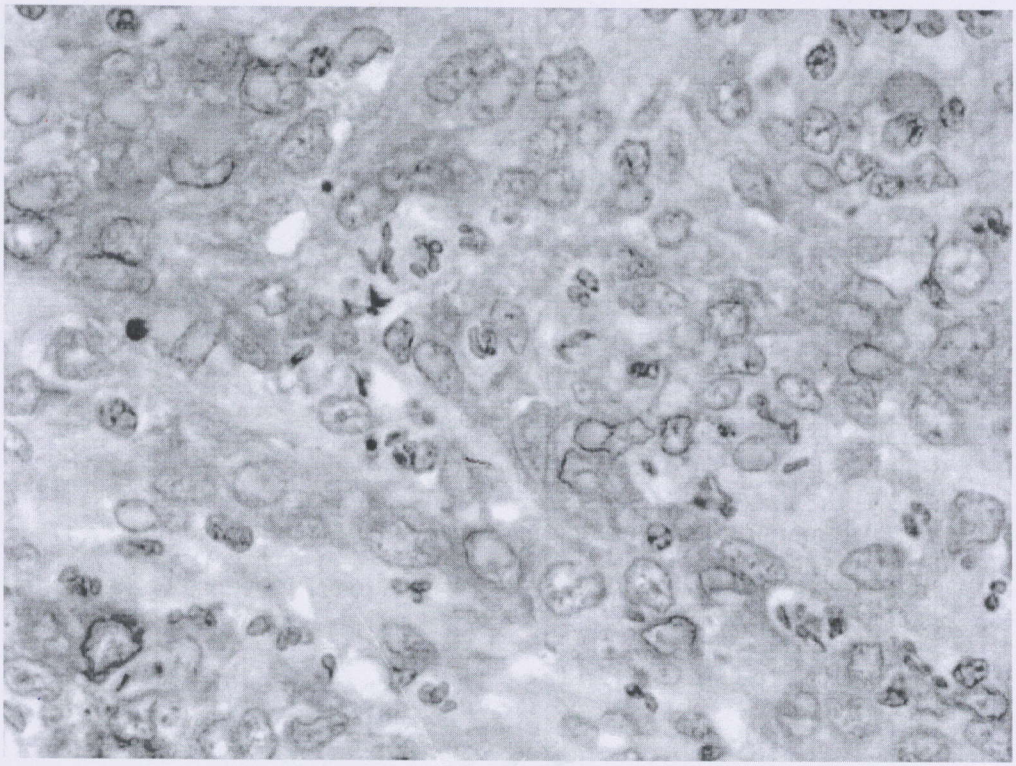


Luminal reactivity of COX-2 in a case of malignant myoepithelioma



Moderate to strong reactivity of COX-2 in the inflammatory foci in a case of carcinosarcoma.

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3. Additional studies on diagnostic and prognostic markers in the serum and tissues of dogs affected with canine mammary tumour

The present study was conducted on 43 mammary tumour bearing and 6 tumour free female dogs to determine the diagnostic and prognostic markers in serum and tissues. Out of these 43 cases, 42 were malignant mammary tumours and 1 benign (hemangioma). The histopathological classification of malignant mammary tumours revealed 33 cases (78.57%) of carcinoma, 6 (14.29%) of carcinosarcoma, 2 (4.76%) of sarcoma and 1 malignant mast cell tumour (2.38%). The additional histopathological changes including inflammation, epithelial-mesenchymal transition (EMT), tumour infiltrating lymphocytes (TIL), connective tissue invasion, vascular and lymphatic thrombosis/embolism, necrosis and margins were also recorded. The maximum tumours were found to be in grade II (51.22%) and stage 5 (66.67%). The mean values of serum hormones (estradiol, progesterone and prolactin), cytokines (IL8 and TNFα) and surface glycoproteins (CA 15.3 and CEA) in mammary tumour cases were significantly higher than those of controls. Immunohistochemical expression of estrogen receptor β and progesterone receptors was noticed in 89.20% and 71.79% and that of TNFα in 83.3% cases, respectively. HER2 over-expression was noted in 3 cases by immunohistochemistry and in 6 cases by real time PCR. All the canine mammary tumour cases were further classified into two subtypes on molecular basis viz. Luminal B (23/41) and Luminal A (18/41). Serum progesterone showed a positive correlation with serum estradiol and prolactin levels and also between serum estradiol and prolactin. However, other markers showed negative correlation with each other. The Kaplen-Meier survival analysis elucidated poor survival in high grade and advance stage tumours and almost similar mean survival in Luminal A and Luminal B molecular subtypes. Inflammation, tumour infiltrating lymphocytes, tumour thrombo-embolism and unclear margins were the additional histopathological changes recorded in the study. Besides these, studies were also conducted related aspects of CMT

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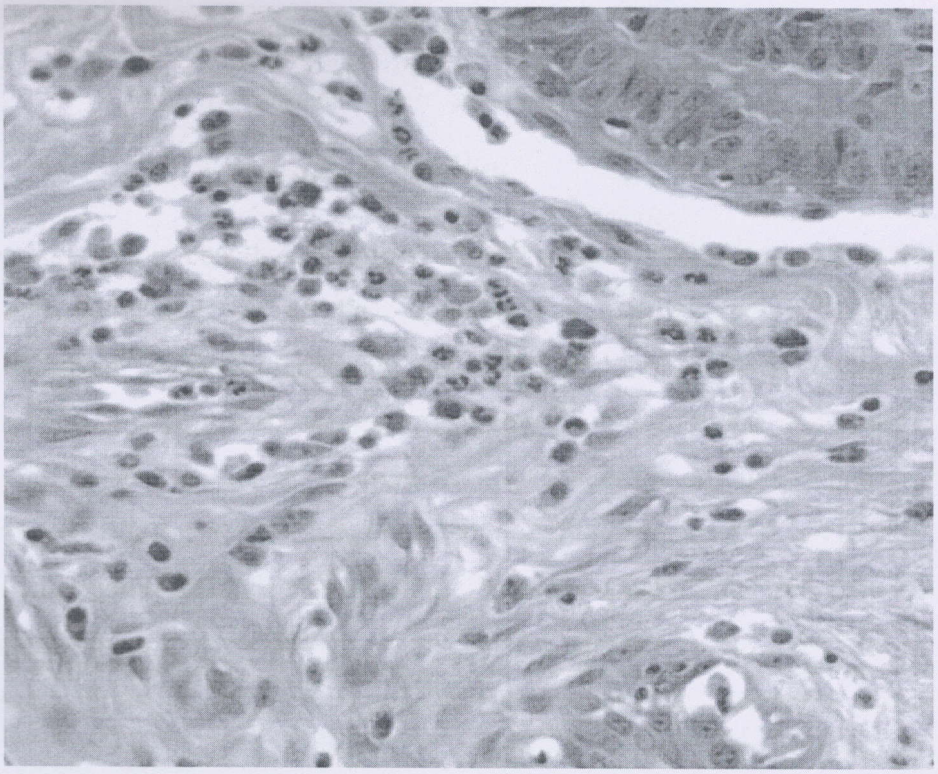
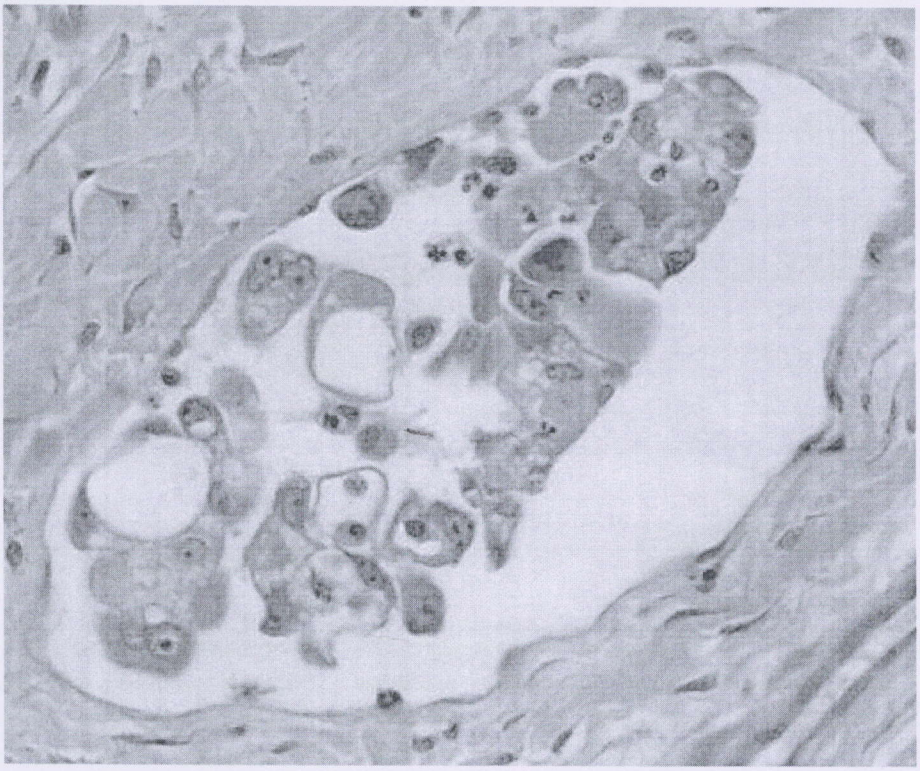


CMT showing inflammation.



CMT revealing lymphatic thrombo-embolism

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Conclusions

It was concluded that a panel of markers should be used for detection of lymphangiogenesis in CMT and it should preferably include podoplanin and PROX 1 as cytoplasmic and nuclear markers, respectively. Peritumoral lymphatics and lymphangiogenesis played a major role in tumour spread although intratumoral neo-lymphangiogenesis was more important in invasion and EMT, besides modulating the tumour microenvironment, therefore both of them appeared to supplement each other. It is suggested that podoplanin and PROX 1 could be potential therapeutic targets for exploring and halting the process of lymphangiogensis in mammary neoplasia of canine and people. It is further added that the role of novel markers of lymphangigenesis viz. COX2, NR2F2 should be explored in future and lymphangiogenetic markers should be comapared with angiogenetic markers and important determinants of cancer spread and metastasis.

Research publications:

S. No.	Author name	Year	Title of publication	Journal name, issue No., Pages	NAAS Rating
1	D S GAVHANE, A SINGH, **N K SOOD and K GUPTA**	2016	Expression of stem cell biomarker aldehyde dehydrogenase 1 (ALDH 1) in canine mammary tumor.	Indian Journal of Animal Sciences 86 (3): 285–287	6.1
2.	Gautam S, **Sood N K and Gupta K.**	2014	Aberrant cytoplasmic accumulation of retinoblastoma protein in basal cells may lead to increased survival in malignant canine mammary tumours.	Veterinarni Medicina, 59 (2): 76–80.	6.68

Paper presented and or abstracts published:

S No.	Year	Authors' name	Title	Symposia/workshops Abstract No. & Page
1.	2014	Gautam S*, **Sood N K.**, Gupta K, Joshi C, Rampal S and Mohindroo J.	Pesticides as potential risk factor in canine malignant mammary tumors with special reference to aromatase induction.	In compendium of XXXI Annual Conference of Indian Association of Veterinary Pathologists and 5th Annual meeting of Indian College of Veterinary Pathologists, Organized by Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University Anand, Gujarat, India, w. e .f. November 13-15, 2014, pp-175, (Technical Session IX- Poster Presentation), Abstract No.18.

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	2014	**Gupta K, Sood N K.** and Singh A.	Canine mammary cancer stem cells reside in the basal epithelium as revealed by single and multiple immunolabelling.	In compendium of XXXI Annual Conference of Indian Association of Veterinary Pathologists and 5th Annual meeting of Indian College of Veterinary Pathologists, Organized by Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University Anand, Gujarat, India, w. e .f. November 13-15, 2014, pp-184, (Technical Session IX- Poster Presentation), Abstract No.39.

Awards and honors related to project work

- **Sood N K, Gupta K,** Singh A and Raghunath M **Team leaders awarded Drs. Nemi Chand Jain and Jawahar Lal Vegad Award for Outstanding Research in Veterinary Pathology on the topic** "Diagnostic, prognostic and predictive evaluation of canine mammary neoplasia as a model for human breast cancer," during XXX Annual Conference of Indian Association of Veterinary Pathologists, organized by Department of Veterinary Pathology , College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture & Technology, Bhubaneswar, Odhisa, India, w. e. f. 21-23 November, 2013.
- **Dr. N K Sood given the Honour to present inaugural key note address** on 'Canine mammary tomor as a model for breast cancer' and co-chairman of inaugural session of the second Indo Global Veterinary submit and expo at Hydrabad in 2015.
- **Dr. N K Sood nominated as Chairman of one the oncology sessions during the Global Cancer Summit held at Bangalore in 2015**

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