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Immunohistochemical evaluation of CD4+ and CD8+ tumour infiltrating lymphocytes and their prognostic significance in canine mammary tumours

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Abstract

Tumour-infiltrating lymphocytes (TILs) are critical components of the tumour microenvironment (TME), comprising T cells, B cells and natural killer cells that influence tumour progression and clinical outcome. Among these, CD4+ and CD8+ T cells play pivotal roles, with CD4+ cells regulating immune responses and CD8+ cells mediating cytotoxic effects. Their distribution between stromal and intratumoural regions holds prognostic significance. The objective of the present study was to investigate the localisation of TILs in relation to histological grade, as well as to evaluate the prognostic relevance of the CD4+/CD8+ ratio. Immunohistochemistry was performed on 25 tumour samples, which revealed abundant CD4+ and CD8+ TILs in grade I tumours, which declined as grade advanced, whereas CD4+ T cells showed a slight predominance in high-grade tumours. Lymphocytes were primarily confined to stromal regions, followed by peritumoural areas, while intratumoural localisation was consistently sparse. Importantly, the CD4+/CD8+ ratio, increased with tumour grade, reflecting a relative predominance of CD4+ T cells in high-grade tumours and suggesting progression towards an immunosuppressive microenvironment. Kaplan–Meier survival analysis revealed that dogs with a low CD4+/CD8+ratio exhibited more favourable overall survival compared to those with a higher ratio ($p< 0.05$). The ratio between helper and cytotoxic T lymphocytes within the TME serves as an important determinant of disease outcome.

Keywords: *Canine mammary tumours, tumour infiltrating lymphocytes, immunohistochemistry, CD4+/CD8+ ratio*

Cancer is a life-threatening condition that poses significant challenges in veterinary medicine. Among dogs, malignant neoplasms account for the highest proportion of deaths (46.3%), followed by ageing-related causes (18.2%) (Dias-Pereira, 2022). Canine mammary tumours (CMTs) are among the most frequently diagnosed neoplasms in intact female dogs and a considerable proportion display malignant behaviour. Data from the Swiss Canine Cancer Registry (2008–2020) indicated that mammary tumours accounted for 14.50% of all reported tumours, ranking third after skin and soft tissue neoplasms (Dhein *et al.*, 2024). Similarly, a three-year survey conducted at the University Veterinary Hospitals in

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Thrissur, Kerala, recorded 265 superficial neoplasms, with CMTs comprising the largest share (51.7%), highlighting their high prevalence in this region (Nair, 2021).

Tumour-infiltrating lymphocytes (TILs) are key players in cancer, as they can regulate tumour growth and progression. Their effect, whether protective or supportive of tumour development, is determined by the surrounding TME (Whiteside, 2022). Among the different subsets of TILs, CD4+ helper T cells and CD8+ cytotoxic T cells are the most prominent. The CD8+ cells directly destroy neoplastic cells, while CD4+ cells coordinate immune functions (Fridman *et al*., 2012); however, certain CD4+ subsets, such as regulatory T cells, can dampen anti-tumour immunity and support tumour survival. The distribution of CD4+ and CD8+ TILs within the tumour is considered important, as their location in either the tumour nests or the surrounding stroma can influence the effectiveness of the anti-tumour immune response. The objective of the present study was to investigate the localisation of TILs in relation to histological grade, as well as to evaluate the prognostic relevance of the CD4+/CD8+ ratio.

Materials and methods

Sample: A total of 25 mammary tumour samples were collected for the present study. Among these, 24 were obtained as excisional biopsy specimens from the Teaching Veterinary Clinical Complex (TVCC), Mannuthy and the University Veterinary Hospital, Kokkalai. In addition, one necropsy specimen was collected from the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy.

Histopathology**:** All samples were fixed in 10% neutral buffered formalin (NBF) and processed for histopathology and immunohistochemistry. The tissue samples were processed using routine histopathological procedures and subsequently stained with haematoxylin and eosin (H&E) for histopathological evaluation (Suvarna *et al.*, 2019).

Classification and histological malignancy grading of CMTs: The classification of CMTs was performed according to the criteria of Goldschmidt *et al.* (2011), while grading of malignancy was based on the system of Clemente *et al.* (2010). Accordingly, tumours were categorised into grade I (well differentiated), grade II (moderately differentiated) and grade III (poorly differentiated).

Immunohistochemistry: Immunohistochemistry was performed on paraffin-embedded tissue sections to localise CD4+ and CD8+ TILs. Sections of 4 µm thickness were mounted on poly-L-lysine coated slides and subjected to antigen retrieval using citrate buffer (pH 6.0) in a microwave oven for 15 minutes. Endogenous peroxidase activity was quenched with 3 per cent hydrogen peroxide and non-specific binding was reduced by applying 3 per cent bovine serum albumin as protein block. The sections were incubated overnight at 4°C with polyclonal rabbit anti-CD4 and anti-CD8 primary antibodies procured from Origin Diagnostics and Research, Kerala, India at a dilution of 1:50. Subsequently, slides were treated with a secondary antibody (PolyQ stain 2 step detection kit, DSK-211-015, Quartett, Berlin, Germany) and the immunoreactivity was visualised using a DAB chromogen system. Finally, the sections were counterstained with haematoxylin, dehydrated, cleared and mounted for microscopic examination. For negative controls, the primary antibody was replaced with antibody dilution buffer.

IHC scoring: The CD4+ and CD8+ TILs were counted in the intratumoural, stromal and peritumoural regions of the tumour across ten high-power fields (HPF). Immunostaining was evaluated using a combined score that included both the intensity and the number of positive cells. Based on this score, cases were grouped as weak (+), moderate (++) or strong (+++) immunostaining (Vakkala *et al.*, 1999). The CD4+/CD8+ ratio for each case was then calculated from the average number of TILs per field for both markers.

Statistical analysis: The data obtained from the study were analysed using SPSS software (version 24.0). The association of CD4+ and CD8+ expression with tumour grade, localisation within the tumour and their interaction was evaluated using repeated measures ANOVA. The relationship between the CD4+/CD8+ ratio and tumour grade were assessed by the Kruskal–Wallis test. Overall survival was estimated using the Kaplan–Meier method and comparison with CD4+/CD8+ ratio was performed using the log-rank test. Survival time was defined as the period from the date of diagnosis (surgery) to the date of death or last follow-up.

Results and discussion

The study population comprised 25 female dogs with mammary tumours, most frequently Labrador Retrievers, followed by Pomeranians, Dachshunds, crossbred, German Shepherd, Rottweiler, Siberian Husky and non-descript dogs, aligning with the predominance of Labradors reported by Bhimani *et al.* (2024). The age ranged from 3.5 to 16.5 years, with most cases in middle-aged to older dogs, consistent with the reports of Burrai *et al.* (2022). Tumour sizes varied between 1.83 and 17.2 cm, comparable to the distribution described by Śmiech *et al.* (2023), who noted a higher proportion of tumours ≥ 5 cm and fewer cases below 3 cm diameter.

Classification and grading of CMTs

All 25 canine mammary tumours examined were malignant, comprising nine grade I, 10 grade II and six grade III cases. Dolka *et al.* (2018) similarly

Table 1. Histological classification and grading of canine mammary tumours (n = 25)

Tumour type	Grade I (n)	Grade II (n)	Grade III (n)	Total (n)
Cystic papillary carcinoma	2	0	0	2
Intraductal papillary carcinoma	2	0	0	2
Tubulopapillary carcinoma	2	3	0	5
Adenosquamous carcinoma	2	0	2	4
Ductal carcinoma	1	1	0	2
Malignant myoepithelioma	0	2	0	2
Carcinoma *in situ*	0	1	0	1
Carcinoma mixed type	0	1	0	1
Tubular carcinoma	0	1	0	1
Solid carcinoma	0	1	1	2
Comedocarcinoma	0	0	1	1
Anaplastic carcinoma	0	0	1	1
Micropapillary carcinoma	0	0	1	1
Total	**9**	**10**	**6**	**25**

reported a predominance of grade II carcinomas (38.2%, 215/562). Grade I included cystic papillary, intraductal papillary, tubulopapillary, adenosquamous and ductal carcinomas, while grade II tumours were more varied, with tubulopapillary being the most common, along with malignant myoepithelioma and isolated cases of other subtypes. Grade III consisted of adenosquamous, solid, comedocarcinoma, anaplastic and micropapillary carcinomas. Comparable to the present study, Chu *et al.* (2012) also reported a higher occurrence of tubulopapillary carcinoma in dogs, which is generally associated with a better prognosis. Histopathology further revealed lymphocytic infiltration within the TME (Figs. 1. and 2.).

Immunohistochemistry

The distribution of TILs was evaluated across different tumoural regions and categorised as weak, moderate or strong based on the intensity of staining and the number of positive cells (Fig. 3. to Fig. 8.) and corresponding scores of 1, 2 and 3 were assigned, respectively.

Comparison of expression of CD4+ and CD8+ TILs score with tumour grades in CMTs

In the present study tumour grade was significantly associated with CD4+ and CD8+ TILs ($p < 0.001$) as shown in Table 2. Grade I tumours exhibited the highest infiltration of CD4+ (2.00 ± 0.08) and CD8+ (2.15 ± 0.08) TILs, which was significantly higher compared with grade II and grade III tumours. Grade II tumours showed a moderate level of infiltration, while grade III tumours displayed the lowest expression of both subsets (CD4+: 1.00 ± 0.10; CD8+: 0.94 ± 0.10).

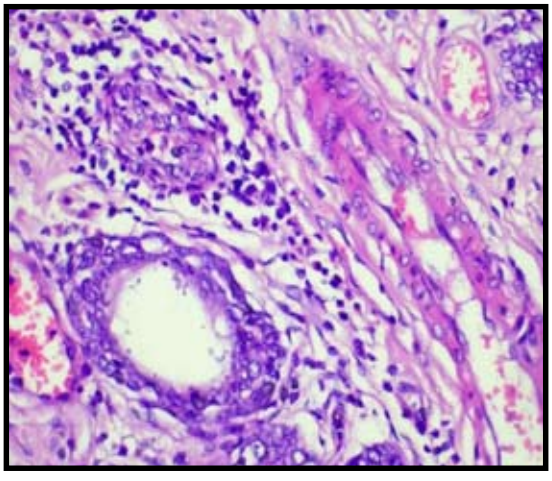

Fig. 1. CMT-Ductal carcinoma of the mammary gland showing few intratumoural lymphocytes but a more prominent stromal lymphocytic infiltration (H&E × 400).

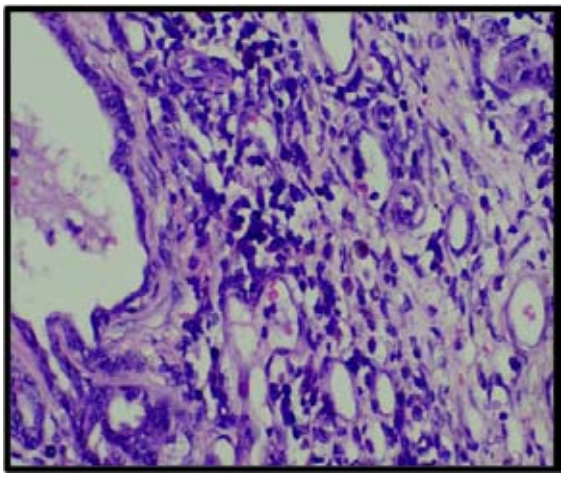

Fig. 2. CMT-Tubular carcinoma showing prominent stromal lymphocytic infiltration. Numerous small, round, darkly stained lymphocytes are concentrated within the stroma (H&E × 400)

When comparing CD4+ and CD8+ TILs expression within each tumour grade, in grade I tumours CD8+ expression was marginally higher than CD4+ expression. In grade II tumours, both subsets demonstrated identical mean values. In grade III tumours, CD4+ levels were slightly higher than CD8+ levels. Thus, although CD8+ predominated in grade I and CD4+ in grade III, the overall trend showed a parallel decline of both subsets with advancing tumour grade.

These findings align with Estrela-Lima *et al*. (2010) and Astuti *et al*. (2022), who reported higher CD8+ TILs in CMTs and grade I invasive breast carcinomas respectively, with reduced infiltration in high-grade tumours, indicating a weakened cytotoxic response. The predominance of CD4+ cells in high-grade tumours, as seen in this study, may indicate shifts in CD4+ subsets, consistent with Huang *et al.* (2015), who reported enrichment of regulatory T cells and Th17 cells in advanced tumours. Together, these mechanisms explain the decline in CD8+ TILs and the predominance of CD4+ T cells observed in high-grade tumours, contributing to the development of a pro-tumour microenvironment.

Table 2. Comparison of CD4+ and CD8+ TILs score with tumour grades in CMTs

Tumour grade	CD4+ TILs (Mean ± SE)	CD8+ TILs (Mean ± SE)	Overall p-value
I	2.00 ± 0.08[a]	2.15 ± 0.08[a]	<0.001**
II	1.27 ± 0.08[b]	1.27 ± 0.08[b]	
III	1.00 ± 0.10[c]	0.94 ± 0.10[c]	

***significant at 0.01 level; Means having different superscripts (small letters a-c within columns) differ significantly*

Comparison of expression of CD4+ and CD8+ TILs with locations within the tumour

The distribution of CD4+ and CD8+ TILs in the present study revealed that both subsets were least abundant intratumourally and more prevalent in stromal and peritumoural regions (Figs. 9. and 10.).

Evidence from human cancers further supports the prognostic value of stromal lymphocytes. In breast cancer, stromal TILs, particularly CD8+ subsets, have been shown to independently predict survival, with reduced stromal infiltration correlating with poorer outcomes (Vihervuori

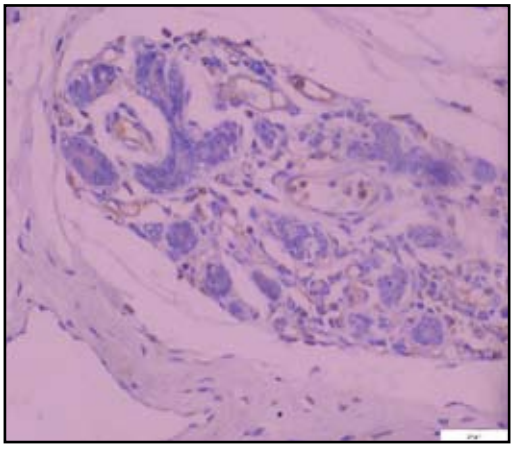

Fig. 3. CMT-Adenosquamous carcinoma- Weak positivity for CD4+ TILs (IHC x 400)

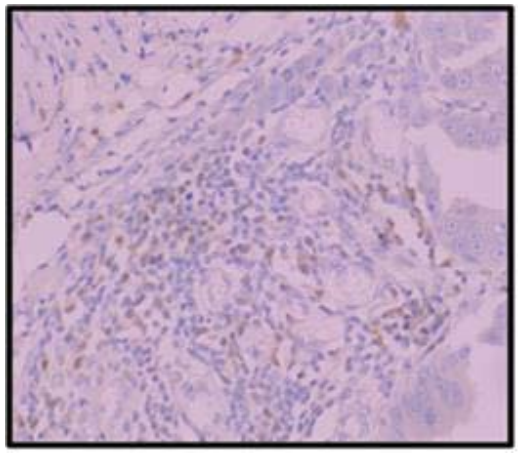

Fig. 4. CMT-Carcinoma in-situ - Weak positivity for CD8+ TILs (IHC x 400)

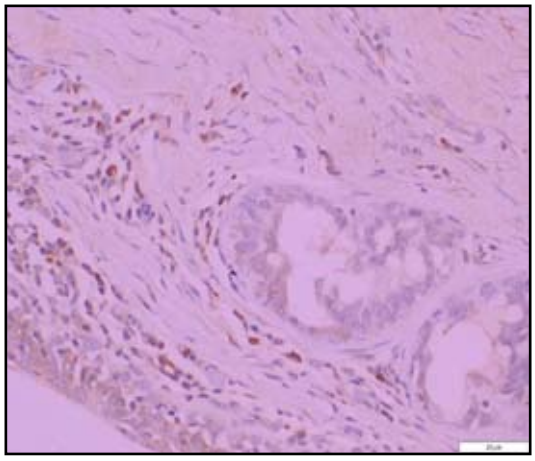

Fig. 5. CMT-Cystic papillary carcinoma- Moderate positivity for CD4+ TILs (IHC x 400)

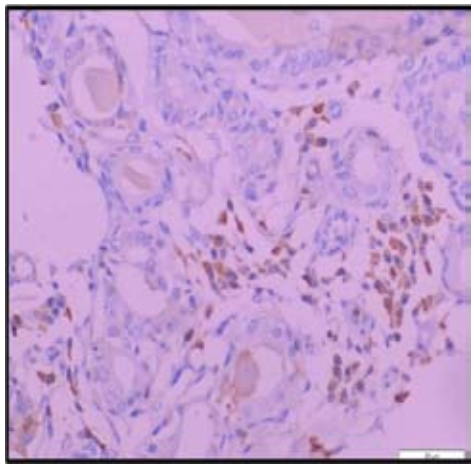

Fig. 6. CMT-Ductal carcinoma- Moderate positivity for CD8+ TILs (IHC x 400)

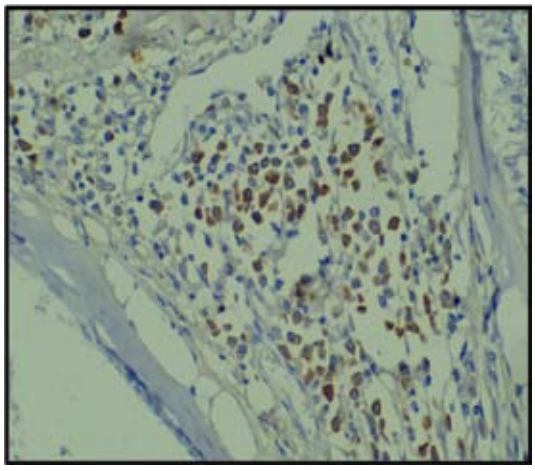

Fig. 7. CMT-Tubulopapillary carcinoma- Strong positivity for CD4+ TILs (IHC x 400)

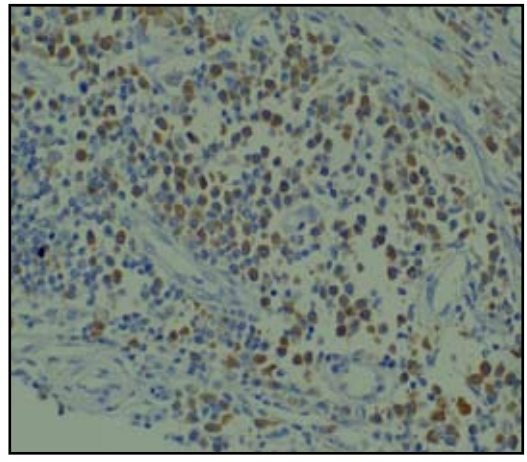

Fig. 8. CMT-Tubulopapillary carcinoma- Strong positivity for CD8+ TILs (IHC x 400)

Table 3. Comparison of expression of CD4+ and CD8+ TILs with location within the tumour

Tumour compartment	CD4+ TILs (Mean ± SE)	CD8+ TILs (Mean ± SE)	p-value (CD4+ across locations)	p-value (CD8+ across locations)
Intratumoural	1.296 ± 0.041[b]	1.278 ± 0.040[b]	0.079[ns]	0.012[*]
Stromal	1.504 ± 0.079[a]	1.615 ± 0.094[a]		
Peritumoural	1.467 ± 0.068[a]	1.467 ± 0.068[a]		

**Significant at 0.05 level; ns non-significant. Means having different superscripts (small letters a-b within columns) differ significantly*

et al., 2019; Koletsa *et al*., 2020). Since stromal TILs are considered more consistent and reliable than intratumoural TILs, which are often sparse and unevenly distributed (Salgado *et al*., 2015), the predominance of stromal and peritumoural lymphocytes in the present study highlights their diagnostic and prognostic significance. Reduced intratumoural infiltration of TILs in aggressive tumours may be explained by immune exhaustion and suppression within the TME (Soudja *et al*., 2010). These factors together likely contribute to the decreased intratumoural and overall infiltration observed in high-grade tumours.

CD4+ and CD8+ T-cell infiltration across tumour grades and locations

The analysis of CD4+ and CD8+ TILs across tumour grades and locations within the tumour revealed distinct changes in their distribution with disease progression as shown in table 4.

These findings highlight a progressive decline in lymphocytic infiltration with increasing grade, consistent with a shift from an immune-active to an immunosuppressed microenvironment.

In contrast, some studies have reported higher TIL density in high-grade tumours, often associated with aggressive histological features and lymphatic invasion (Kim *et al*., 2013; Flecher *et al*., 2025). The reduced infiltration observed in the present study may instead reflect tumour-driven immune evasion. Multiple mechanisms can limit immune cell entry into tumour nests. Structural barriers such as abnormal vasculature and dense extracellular matrix (Chung *et al*., 2021), loss of T-cell homing ligands and the release of immunosuppressive factors (Melssen *et al*., 2023; Rezaie *et al*., 2024) are important contributors.

In addition to these barriers, functional alterations in T-cell subsets further promote immune suppression. At the same time, chronic antigenic stimulation leads to CD8+ T-cell exhaustion, diminishing cytotoxic activity and weakening tumour control (Dolina *et al*., 2021). Collectively, these mechanisms explain the reduced infiltration and functional impairment of CD4+ and CD8+ subsets in high-grade tumours, contributing to immune escape and tumour progression.

Comparison of expression of CD4+/CD8+ ratio with tumour grades in CMTs

As tumour grade increased from I to III, the average CD4+/CD8+ ratio also increased, with the difference being statistically significant ($p<0.05$) as shown in Table 5. This indicates that higher grades are associated with a relative predominance of CD4+ over CD8+ cells, reflecting alterations in the immune microenvironment during tumour progression. Similar findings were reported by Estrela-Lima *et al*. (2010), who observed that advanced canine mammary carcinomas had higher CD4+/CD8+ ratios and that animals with elevated ratios experienced shorter survival. These results support the prognostic relevance of the CD4+/CD8+ ratio, suggesting that a

Table 4. CD4+ and CD8+ T cell infiltration across histological grades and locations within the tumour

Marker	Grade	Intratumoural	Stromal	Peritumoural	p value (Grade x location)
CD4+	I	1.89 ± .11^{a}	2.11 ± .11^{a}	2.00 ± .00^{a}	0.181
	II	1.00 ± .00^{Bb}	1.40 ± .16^{Ab}	1.40 ± .16^{Ab}	0.013*
	III	1.00 ± .00^{b}	1.00 ± .00^{b}	1.00 ± .00^{c}	1.000
p value (Location x grade)		< 0.000*	< 0.000*	< 0.000*	
CD8+	I	2.00 ± .00^{Ba}	2.44 ± .18^{Aa}	2.00 ± .00^{Ba}	0.002*
	II	1.00 ± .00^{Bb}	1.40 ± .16^{Ab}	1.40 ± .16^{Ab}	0.012*
	III	0.83 ± .17^{b}	1.00 ± .00^{b}	1.00 ± .00^{c}	0.575
p value (Location x grade)		< 0.000*	< 0.000*	< 0.000*	

***significant at 0.01 level; *significant at 0.05 level; ns non-significant. Means having different superscripts (small letters a-c within columns, capital letters A-C within rows) differ significantly*

predominance of CD4+ cells may compromise cytotoxic responses and contribute to tumour progression and poor outcome.

Kaplan–Meier survival analysis

Kaplan-Meier survival analysis of the study cases was carried out to determine the prognosis. Clinical follow up of the cases was done from May 2024 to July 2025. Out of 25 cases, 3 events (deaths) were recorded, while 22 cases (88.0%) were censored. All cases in grade I (n = 9) and grade II (n = 10) were censored, with no deaths observed during the study period (100% survival). In contrast, grade III tumours (n = 6) recorded three deaths, with only three cases censored (50%).

Kaplan–Meier survival analysis based on CD4+/CD8+ ratio

Kaplan–Meier analysis demonstrated a significant association between CD4+/CD8+ ratio and survival. Dogs with a low ratio (≤ 1.05) showed 100% survival with a mean survival period of 13 months, whereas those with a high ratio (> 1.05) experienced reduced survival, declining to 64.9% by five months with a mean survival period of 10.7 months. The log-rank test confirmed this difference ($p < 0.05$), indicating that a predominance of CD4+ over CD8+ cells is linked to poorer outcome. Similar trends have been reported in human breast cancer, where elevated CD4+/CD8+ ratios predicted tumour progression and shorter survival (Yang *et al*., 2017) and in CMTs, where higher ratios correlated with metastasis and reduced survival (Estrela-Lima *et al*., 2010). The adverse effect of a high ratio may reflect expansion of CD4+ regulatory T cells, which suppress CD8+ cytotoxic activity (Goda *et al*., 2022;

Table 5. Comparison of CD4+/CD8+ ratio with histological grade

Grade	CD4+/CD8+ ratio (Mean ± SE)
I	.94 ± 0.49^{b}
II	1.08 ± .11^{b}
III	1.57 ± .17^{a}
p value	0.003*

**Significant at 0.05 level;Means having different superscripts (small letters a-b within columns) differ significantly*

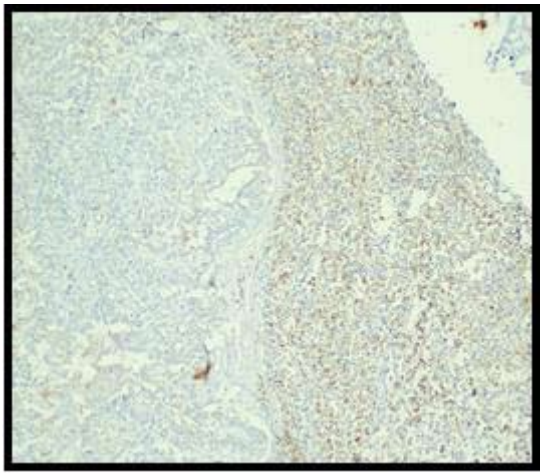

Fig. 9. CMT- Stromal infiltration- Strong positivity for CD8+ TILs (IHC x 100)

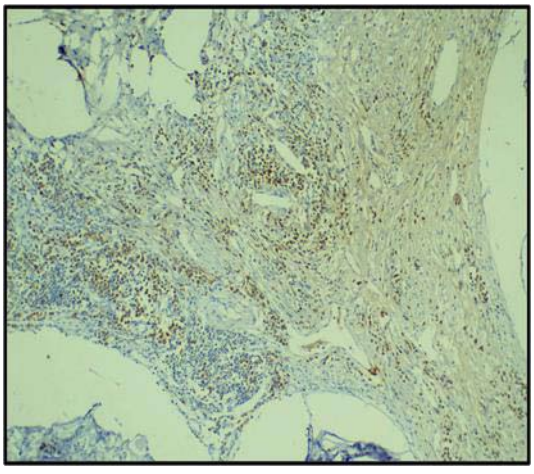

Fig. 10. CMT-Peritumoural infiltration- Strong positivity for CD4+ TILs (IHC x 100)

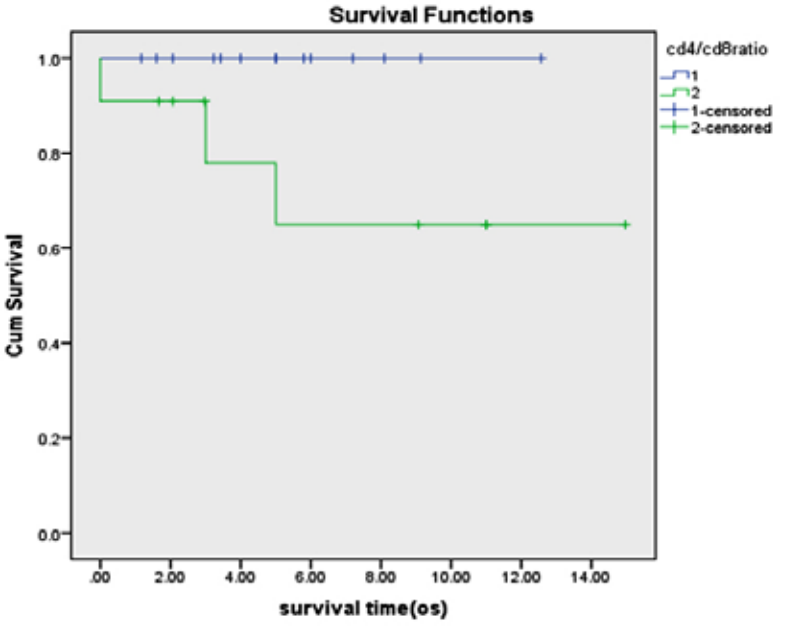

Fig. 11. Kaplan–Meier survival analysis based on the CD4+/CD8+ ratio demonstrated prognostic variation. The y-axis represents cumulative survival probability and the x-axis indicates survival time in months (OS). Dogs with a low ratio (Group 1, blue curve) maintained higher survival throughout the study, whereas those with a high ratio (Group 2, green curve) exhibited a progressive decline with earlier events. Censored cases are marked by crosses and the results suggest that a lower CD4+/CD8+ ratio is associated with better prognosis in canine mammary tumours.

Rech *et al*., 2010), thereby promoting immune evasion and tumour progression and needs further investigations.

Conclusion

This study demonstrated that CD4+ and CD8+ TIL distribution and ratios are strongly associated with tumour grade and prognosis in CMTs. Low-grade tumours were characterised by high infiltration of both subsets, particularly stromal CD8+ cells, indicating a favourable cytotoxic response. In contrast, high-grade tumours showed markedly reduced infiltration, a predominance of CD4+ over CD8+ cells and an elevated CD4+/CD8+ ratio, all correlating with poor survival. Stromal and peritumoural compartments emerged as the principal sites of prognostic immune activity, mirroring findings in human cancers. Overall, the CD4+/CD8+ ratio, together with the spatial distribution of TILs, may serve as a valuable prognostic marker in CMTs. Future investigations exploring the functional dynamics of different T helper subsets and mechanisms of T cell exhaustion may provide additional insights into tumour immune regulation and aid in the development of targeted immunotherapeutic strategies in CMTs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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