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# High lysyl oxidase expression is an indicator of poor prognosis in dogs with cutaneous mast cell tumours

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## Abstract

Mast cell tumour (MCT) is one of the most frequent skin tumours in dogs. Due to their unpredictable biological behaviour, MCTs often cause several therapeutic frustrations, leading to investigation regarding prognostic markers. Lysyl oxidase (LOX) is an enzyme that promotes extracellular matrix stability and contributes to cell migration, angiogenesis and epithelial-mesenchymal transition. Its expression positively correlates with poor prognoses in several human and canine mammary cancers. The aim of this study was to characterise the immunohistochemical expression of LOX in MCT samples and compare it with histological grading and post-surgical survival. Twenty-six tumours were submitted to immunohistochemistry for LOX expression evaluation. All samples were positive for LOX, with variable percentages of cytoplasmic and nuclear positivity. Cytoplasmic positivity was significantly higher in high-grade MCTs ($P = .0297$). Our results indicate that high expression of cytoplasmic LOX in neoplastic mast cells is an indicator of poor prognosis for canine cutaneous MCTs.

**KEYWORDS**
dogs, extracellular matrix, immunohistochemistry, lysyl oxidase, mastocytoma, prognosis

## 1 | INTRODUCTION

Cutaneous mast cell tumours (MCTs) comprise almost one-fourth of all malignant tumours diagnosed in dogs and their biological behaviour is extremely variable, making it very difficult to assess the degree of malignancy merely by macroscopic examination.[1–3] Although the aberrant expression of the *kit* gene has been proposed as a major aetiological factor for the disease, in at least 60% of the cases mutations in such gene is not present, indicating MCTs have a multifactorial and still poorly understood aetiology.[1–4]

Elder dogs and brachycephalic breeds have a particular predisposition to the disease.[1–3] Pugs and Boxers usually have less aggressive MCTs, with a more favourable prognosis, while Shar-Peis and Labradors, on the other hand, frequently present high-grade MCTs.[2,3] The definitive diagnosis of canine MCTs can be achieved by cytology or histopathology. Tumour staging and documentation of paraneoplastic signs are needed in order to elect the best therapeutic approach.[3] Histologic grading according to the Patnaik and Kiupel systems is also a very important diagnostic tool for canine MCTs, since the degree of cellular differentiation influences tumour behaviour, progression and aggressiveness.[1–6]

  

Therapeutic frustrations caused by the unpredictability of canine MCTs has led to intense research for more reliable prognostic indicators. Currently, the main criteria for prognostic evaluation of canine cutaneous MCTs are histological grading, mitotic index, Ki67 index and pattern of KIT immunostaining.[5–9]

The extracellular matrix (ECM) consists of an elaborate three-dimensional network of macromolecules responsible for maintaining tissue homeostasis through fundamental physical support, preserving functional and mechanical integrity of tissues.[10,11] Acting as a reservoir of several growth factors and biologically active molecules, the ECM is capable of modulating a broad spectrum of cellular functions such as differentiation, cell growth, adhesion, proliferation, migration and apoptosis.[12,13] ECM's biochemical and biomechanical properties are made to suit the needs of the cells, tissues and regions to which it belongs. However, interactions between ECM and surrounding cells are capable of altering cellular behaviour, promoting changes in the extracellular environment.[10–14]

Elastin is an extremely dynamic and flexible fibrous protein that is frequently associated with collagen. It originates elastic fibres, structures that confer elasticity to the ECM and are basically composed of two elements: an amorphous form of elastin in the centre and an outer mantle composed of glycoprotein microfibrils.[15–17] This amorphous form of elastin is obtained from the crosslinking of several soluble secreted tropoelastin molecules, the elastin precursor protein, which becomes insoluble and stable by the enzyme lysyl-oxidase (LOX) or other members of this family (LOXL1 to LOXL4).[15–18]

Tumour behaviour and development of malignant neoplasms are directly related to the ability of cancer cells to influence their microenvironment, modifying local stroma and vascularization.[19,20] Through microenvironmental modifications, cancer cells impair ECM constitution and stability, causing the modified microenvironment to moderate proliferative activity and tumour invasive behaviour. In addition, the destruction of ECM carried out by several extracellular proteinases is a crucial process for cell invasion and tumour metastasis.[21,22]

The lysyl-oxidase family of enzymes is composed of six members, LOX and LOXL1 to LOXL5. Based on overall structure and sequence similarity, those six enzymes can be grouped into two subfamilies: (i) LOX, LOXL1 and LOXL5; and (ii) LOXL2, LOXL3 and LOXL4. It is noteworth, however, that LOXL5 is not found in mammals. The main physiological function of the lysyl-oxidases is to promote ECM structural stability through catalytic covalent crosslinking of collagen and elastin.[23–25]

In humans, LOX gene is located at chromosome 5 and transcribes a 48 kDa intracellular precursor and a 32 kDa extracellular protein composed of 417 amino acids, which is involved in several biological processes, such as cell migration, angiogenesis, epithelial-mesenchymal transition, tumour cells aggressiveness and metastatic invasion.[26–29] Canine LOX gene has been mapped on chromosome 11 and seems to be involved in the same biological processes as the human protein.[30,31]

The aim of the present study was to characterise the immunohistochemical expression of LOX in MCT samples and compare it with histological grading and post-surgical survival.

## 2 | MATERIALS AND METHODS

Twenty-six samples of canine cutaneous MCTs were selected from the Tumour Bank of the Laboratory of Compared and Translational Oncology at Faculty of Animal Science and Food Engineering, University of São Paulo, Brazil (LOCT-FZEA-USP). Five (5/26, 19%) tumours were grade I, fifteen (15/26, 58%) grade II and six (6/26, 23%) grade III, according to Patnaik system; and seventeen (17/26, 65%) were low-grade and nine (9/26, 35%) high-grade according to Kiupel system. The tumours were graded by an experienced veterinary pathologist (RFS). The criteria for inclusion of the cases were: dermal/cutaneous MCTs, minimum clinical follow-up for censored cases of 180 days after tumour surgical excision aiming the cure, with wide surgical margins, and no other treatment modality associated. Dogs that were alive at the end of the study and those whose death were not related to the disease were censored.

Samples were processed histologically according to routine paraffin embedding techniques. Antigen retrieval was achieved by heating the slides in citrate buffer (pH 6.0) for 25 min at 95°C in a steamer. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide solution for 1 h, and nonspecific interactions were prevented with 5% skimmed milk solution for 1 h. The slides were incubated with a primary rabbit polyclonal antibody anti-LOX (PA1-46020, ThermoFisher, dilution 1:500) for 1 h at room temperature. Cross-reactivity of the primary antibody with the canine LOX was confirmed in our laboratory through Western blot analysis. Secondary antibody (LSAB + System-HRP, Easylink ONE, Easypath) was applied for 25 min according to manufacturer's specifications. Labelling was revealed with DAB chromogen and sections were counterstained with Harris's haematoxylin. Negative control slides were incubated with normal rabbit IgG in the same concentration and conditions of the primary antibody. Slides of human breast carcinoma were also used as positive and negative controls, in addition to canine mammary samples.

Five high-power field digital images per case were obtained with a microscope coupled to a digital camera (Leica DM500 and Leica ICC50HD, Leica Microsystems, Heerbrugg, Switzerland), using the 40x objective (area of each image = 0.08 $mm^2$). The fields were selected from areas with higher frequency of immunolabelling ("*hot spots*"). The percentage of positive nuclei and/or cytoplasms was determined using the ImageJ software (NIH, USA) and compared with histological grading using analysis of variance (ANOVA)/Kruskal-Wallis test followed by Dunn's multiple comparisons test or Mann–Whitney test. Survival analysis was performed using the Kaplan–Meier method followed by log-rank test. Statistical analyses were performed using GraphPad Prism (version 7.0a for MacOS, GraphPad Software Inc.) and Bioestat (version 5.0, Universidade Federal do Pará, PA, Brazil) and significance level was set as 5%.

The Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil, approved the procedures performed on the present study (CEUA 6686250717). Cell line validation statement: no cell line was used in this study.

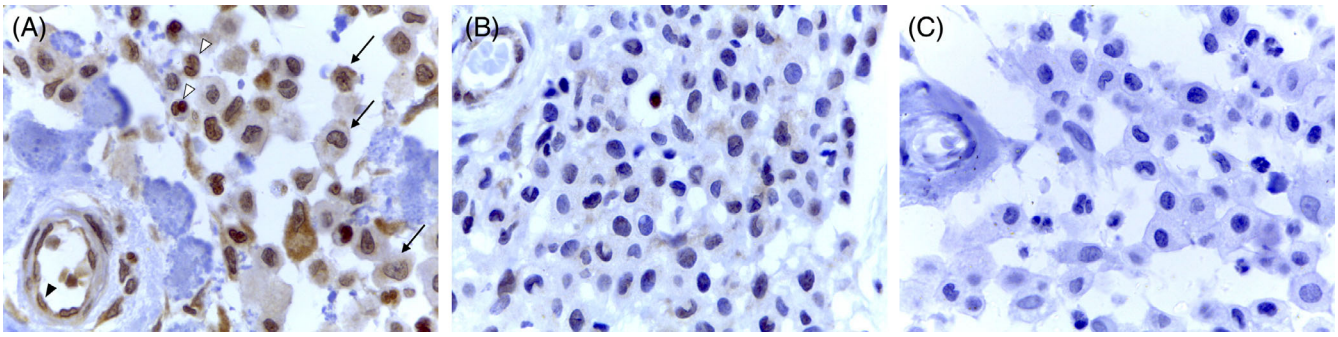


**FIGURE 1** Mast cell tumour immunostaining for LOX. Photomicrographs showing (A) high percentage of LOX nuclear and cytoplasmic immunolabelling in mast cells (arrows), endothelial cells (black arrowhead) and eosinophils (white arrowhead), (B) low percentage LOX nuclear and cytoplasmic immunolabelling and (C) negative control for LOX. IHC, Counterstained with Harris's haematoxylin.

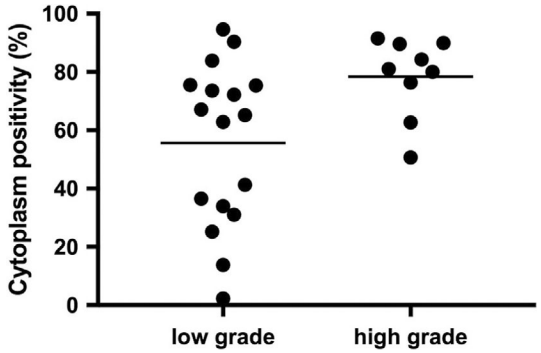


**FIGURE 2** Cytoplasmic positivity percentage for LOX in neoplastic mast cells of low and high grade MCTs, according to the Kiupel's grading system (Unpaired t test, $P = .0297$).

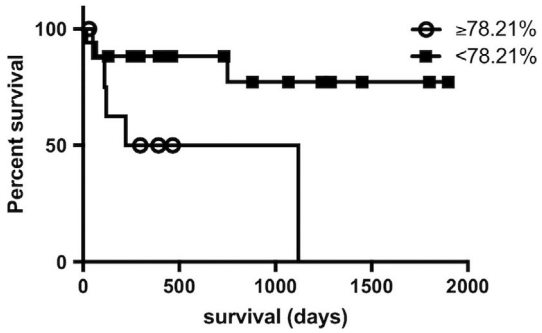


**FIGURE 3** Survival curves for dogs with low (<78.21%) and high (≥78.21%) cytoplasmic positivity for LOX (Kaplan–Meier followed by log-rank test; $P = .0196$, $\chi^2 = 5.45$). Points indicate censored events.

## 3 | RESULTS

The mean age of the dogs was 9 years old and 17 were males (65%). Study population consisted of nine different breeds: mongrel dogs (9/26, 35%), Labrador Retriever (4/26, 15%), Boxer, Dachshund, Poodle (3/26, 11% each), American Pit Bull, Brazilian Mastiff, Doberman and Shih Tzu (1/41, 4% each). Twenty-three dogs presented 1 tumour and 3 dogs had 2 tumours. Most of the MCTs were found on the limbs (11/26, 42%), followed by thorax (8/26, 31%), inguinal region (3/26, 11%), abdomen (2/26, 8%), and head and neck (2/26, 8%). The clinical follow-up varied from 17 to 1898 days post-surgery. Of the 26 dogs, 8 died due to the disease (31%), 4 died for other reasons (15%), and 14 were still alive by the end of the follow-up (54%).

All samples were positive for LOX, with variable percentages of nuclear and cytoplasmic positivity in neoplastic mast cells (Figure 1). Other cells such as endothelial cells, fibroblasts and leukocytes were also variably positive. The average percentages for nuclear and cytoplasmic positivity were 65.64% ± 22,17% (mean ± SD) and 57.38% ± 36.91%, for grade I tumours; 56.17 ± 22.10% and 60.72% ± 25.41%, for grade II tumours; and 71.95% ± 26.36% and 75.61% ± 15.91% for grade III tumours, respectively.

The average percentages for nuclear and cytoplasmic positivity were 56.30% ± 22.03% and 55.59% ± 27.79%, for low-grade tumours; and 72.62% ± 22.41% and 78.48% ± 13.67% for high-grade tumours, respectively. Cytoplasmic positivity was significantly higher in high-grade MCTs ($P = .0297$) (Figure 2), while no statistically significant differences were found for nuclear positivity, or between grades using the Patnaik system. A cut-off value of 78.21% for cytoplasmic positivity was established using a ROC Curve and survival analysis revealed that dogs with higher percentage of positive cells had significantly shorter survival ($P = .0197$, median survival = 670 days) (Figure 3).

## 4 | DISCUSSION

The results presented herein show that cytoplasmic LOX expression in neoplastic mast cells is higher in high-grade canine cutaneous MCTs. Moreover, LOX expression is an indicator of shorter survival time for dogs with MCTs in which more than 78.21% of the neoplastic mast cells are positive for the protein.

LOX is an active participant in extracellular matrix metabolism whose overexpression is closely related to the establishment of worse prognosis in several types of human malignancies, such as ovarian, breast, laryngeal, colorectal and gastric cancers; head and neck, oral, liver and lung carcinomas.[26,27,32–35] In veterinary oncology, we found

only one study about LOX expression in canine tumours that proposed LOX as a diagnostic biomarker and prognostic factor for canine mammary tumours.[30]

LOX is produced by several cell types, such as endothelial cells, smooth muscle cells and fibroblasts.[25–27] Its expression has been found to be associated with several cytokines and growth factors and these interactions could help explain its influence on tumour malignancy, promoting ECM transformation, angiogenesis and cell survival. For instance, LOX is capable of inhibiting basic fibroblast growth factor (bFGF)[36] and transforming growth factor β (TGF-β).[37] On the other hand, TGF-β and tumour necrosis factor-alfa (TNF-α) are known to promote LOX mRNA and protein overexpression in cardiac fibroblasts, with the involvement of PI3Kinase/Akt and Smad3 signalling pathways: active complexes related to Smad4 can enter the nucleus and activate LOX promoter region, causing increased expression.[25,38,39] Zhao et al. (2020) demonstrated LOX anti-apoptotic role on TNF-α-treated rat nucleus pulposus cells by suppressing Fas/FasL pathway and p53 phosphorylation at nuclear level.[40] Moreover, one study regarding chronic chagasic cardiomyopathy in mice reported the high expression of both LOX and TIMP-1 associated with fibrosis.[41]

Canine cutaneous MCTs are malignant neoplasms with frequent tissue invasion and metastasis.[42,43] The progression of malignant neoplasms is directly related to the influence of cancer cells on their microenvironment, since stromal modification cause rearrangement of local vascularization and secretion of several biologically active factors.[20,44] In addition, tissue invasion by neoplastic cells depends on the action of proteolytic enzymes capable of degrading ECM, mainly matrix metalloproteinases (MMPs), whose expression has already been identified in canine cutaneous MCTs.[45–47]

The main function of LOX is to promote crosslinking of collagen and elastin.[23–25] Regarding collagen structure, Giantin et al.[46] demonstrated the existence of a positive correlation between MMP-9, a type-IV collagenase, TIMP-2, VEGF-A and histological grade in 35 samples of canine cutaneous MCTs, suggesting their value as indicators of malignancy.[46] Corroborating with these results, our research group demonstrated that dogs with cutaneous MCTs that showed high TIMP-1 expression had longer survival, as well as those with higher intratumoral collagen index.[47,48]

One limitation of the present study was the small number of cases for the survival analysis, although statistically significant differences between low and high-grade MCTs were found. Also, MCTs have been graded by a single pathologist, but the prevalence reported here is similar to a recent study, with the majority of the MCTs been graded as low-grade tumours.[49]

Taken together, the results presented herein reinforce the importance of investigations regarding factors that can modify the ECM structure in cancer. These proteins have great potential to be used as prognostic markers and, possibly, as targets in new treatment modalities. Further studies must be conducted to investigate these hypotheses.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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