Differential Expression of Galectin-1 and Galectin-3 in Canine Non-malignant and Malignant Mammary Tissues and in Progression to Metastases in Mammary Tumors

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Abstract. *Background: Galectin-1 and galectin-3 are carbohydrate-binding proteins that have been implicated in the pathobiology of several types of cancer. The aim of the present study was to investigate the expression pattern of both these galectins in canine non-neoplastic mammary tissues and mammary tumors (CMT). Materials and Methods: Protein and mRNA expression of galectin-1 and -3 were assessed in 12 benign and 41 malignant CMT. Results: Galectin-1 was overexpressed in the majority of malignant CMT cases in tumor cells and stroma. Its expression in malignant tumor cells was associated with smaller-sized tumours. Distant metastases presented a strong intensity of galectin-1 and reduced galectin-3 expression, while the opposite was observed in circulating tumor cells. Interestingly intravascular tumor cells presented galectin-3 up-regulation at the mRNA level. Double-labelling further made it clear that galectin-3 and galectin-1 expression did not overlap in normal-adjacent mammary and CMT cells. Conclusion: Taken together, our data suggest that malignant CMT cell sub-populations have alternating expression of galectin-1 or -3. This might confer survival advantage to tumour cells in different phases of tumour progression.*

Metastases are a frequent complication of neoplasias and the main cause of canine mammary tumour (CMT)-related death. During metastasis, cancer cells gain increased motility due to decreased cell–cell and cell–extracellular matrix (ECM) adhesion and become able to migrate through basement membranes and the ECM. In vessels, in contrast, the ability of cells to aggregate is essential for survival. At distant locations with an appropriate microenvironment, new adhesive interactions will develop, thus facilitating the establishment of metastases (1). Hence, the process of distant metastasis implies a very close interplay and adaptation between cancer cells and the microenvironment both at the primary and distant sites. Cancer in companion animals provides good opportunities to investigate new diagnostic and therapeutic approaches aimed at human cancer (2). CMTs share similarities with human breast cancer which justify their detailed descriptive studies for translation purposes (3).

Galectins 1 and 3 are the most intensely studied members of the galectin family of β-galactoside-binding proteins and have been shown to be differently expressed in several types of tumours and even to play contrasting roles in several types of cancer (4). In experimental settings, galectins have often been implicated in phenomena important in both early and late stages of the metastatic process. These include, among others, cell proliferation (5, 6), tumour angiogenesis (7), tumour-immune escape (8, 9), and tumour invasion through the ECM (10). Both galectins are thought to mediate motility and invasive capacities of cancer cells (11) due to their ability to interact *in vitro* with ligands present in the ECM, such as laminin (12), fibronectin (11) and integrin (13). However, contrasting relations of galectin expression and metastatic features have been reported for different cancer types (14).

In accordance with a pro-tumorigenic function, galectin-1 overexpression in cancer stroma has been associated with

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invasiveness and clinical TNM stage in human breast cancer (15). Moreover, in proteomic studies, it was associated with a metastatic phenotype of a breast cancer cell line xenografted into mice (16), and found to be one of the proteins up-regulated in a highly metastatic variant of another breast cancer cell line (17). In contrast, galectin-1 expression was found to induce apoptosis of colorectal cancer cells (18). Indeed, galectin-1 is known to induce tumour cell death by anoikis in vessels, hindering metastasis (19). Accordingly, galectin-1 down-regulation has also been associated with an acquired tumour resistance to apoptosis-inducing agents (20). A tumor immune escape-promoting effect, on the other hand, has also been attributed to galectin-1 at the site of the primary tumour (21). These findings point to contrasting actions of galectin-1 with respect to the known phases of cancer dissemination. Regarding galectin-3, our group and others have shown that the progression of CMT towards malignancy is associated with a coordinated down-regulation of galectin-3 in tumour cells in primary tumours and loss of its nuclear expression (22,23). However, we also demonstrated a concomitant up-regulation of galectin-3, presumably having an anti-anoikis effect (24), and its binding sites in intravascular tumour cells (22). In a subsequent study, our findings suggested that loss of galectin-3 and sialylation-related masking of its ligand, such as Mucin-1 (MUC1), in conjunction with its overexpression in specific tumour cell sub-populations, are crucial in regulating adhesion in the progression and invasive capacity of metastatic CMT (25). The expression of galectin-3-binding sites was significantly decreased in the ECM of malignant tumours when compared to normal adjacent tissue (22). Despite existing studies documenting the role of galectins in cancer and the finding that galectin-1 and -3 are differentially expressed in human colorectal and prostate cancer (26, 27), such comparative analysis has, to our knowledge, never been investigated in progression of mammary cancer. This prompted us to examine the expression level and localization of both galectin-1 and -3 in spontaneous malignant CMT and their respective metastases, in addition to benign neoplastic and non-neoplastic tissues.

Materials and Methods

Animals. A series consisting of two normal mammary gland samples from female dogs (samples collected during necropsies of non-tumour-bearing animals), 12 benign and 41 malignant CMT was selected from a group of female dogs (of various pure or mixed breeds) which were diagnosed with a single benign or malignant, respectively, mammary gland tumour at the Companion Animal Clinic of Abel Salazar Institute of Biomedical Sciences, University of Porto. The average age of the animals was 10.1 (range=6-14) years. All animals underwent regional or radical mastectomy with curative intent. The malignant CMT-bearing animals were then submitted to a 2-year clinical follow-up, during which they were evaluated every three months by thorough physical examination, abdominal ultrasound and thoracic radiography, to evaluate the post-surgical course of the disease. Whenever distant metastases were detected, they were registered and confirmed by histopathological analysis. The malignant CMTs and 12 randomly selected benign CMTs were previously studied for expression of galectin-3 in tumour cells (22). All tumor samples and normal gland samples were collected with informed consent from pet owners.

Histology. The tumours were fixed in formalin and routinely paraffin embedded. Sections of 3 μm were cut from each paraffin block. All sections were stained with haematoxylin and eosin (H&E) for diagnostic purposes. Histological examination of the tumours was performed independently by two pathologists, and tumours were classified according to the World Health Organization criteria (28). Tumour histological grading and mode of growth was determined as previously described (29).

Immunohistochemistry. Immunostaining was performed using the modified avidin-biotin-peroxidase complex method. A rabbit polyclonal antibody against the *C*-terminus region was used to detect galectin-1 (425800; Invitrogen Corporation, Camarillo, CA, US) and galectin-3 was analyzed using a rabbit polyclonal antibody against the *C*-terminus region (ab31707, Abcam, Cambridge, MA, USA) and a monoclonal antibody (eBioM3/38;eBioscience Inc., San Diego, CA, USA) recognizing its *N*-terminus domain (22). De-paraffinization of 3 μm-thick sections was performed according to routine histological techniques, with Clear-Rite 3 (Thermo scientific, Waltham, MA, US), followed by rehydration in an ethanol/water gradient. Antigen recovery was performed by the immersion of the slides in citrate buffer (pH=6) for 10 min in boiling cycles in a microwave and then slides were cooled off in phosphate buffered saline (PBS) for five minutes. Endogenous peroxidase activity was blocked by treating the slides with 10% H_2O_2 in methanol solution for 10 min. Sections were then incubated with the primary antibody, diluted at 1:150 in PBS containing 5% of bovine serum albumin (BSA), overnight at 4˚C. Afterwards, the slides were washed in PBS. This was followed by incubation with the horseradish peroxidase (HRP)-labelled polymer of the Envision Detection system kit (DAKO, Glostrup, Denmark) for 30 minutes at room temperature and washed in PBS. The reaction was visualized applying 3,3'-diaminobenzidine (DAB) of the same kit for five minutes. All specimens, after washing in running water for five minutes, were lightly counterstained with haematoxylin, dehydrated, and mounted using Clear Rite mounting media. A negative control using 5% BSA instead of the primary antibody was included. As positive controls, human breast carcinoma samples were used. All stained sections were examined with light microscopy and reviewed by three observers (JO, AC, and FG) in the absence of any clinicopathological or patient outcome information. Discrepancies were resolved after discussion at a double-headed microscope.

Evaluation of immunohistochemical data. Galectin-1 positivity, indicated by the presence of brown intracellular or membranous staining, was assessed regarding both the percentage of stained mammary tumour cells and the pattern of immunodetection in both benign and malignant samples. Galectin-1 expression was scored based on the estimation of number of immunoreactive cells. The

percentage of positively stained cells was categorized as follows: fewer than 25% immunostained cancer cells; 25-50% immunostained cancer cells; 51-75% immunostained cancer cells; and more than 75% immunostained cancer cells. Tumour samples were also divided into four groups of galectin-1 expression according to the respective intensity of the stained stromal tissue: weakly-stained=I; moderately-stained=II; strongly-stained=III and very strong staining=IV. For galectin-3 expression, five categories were created: negative, fewer than 10% cell stained; 10-24% immunostained cancer cells; 25 to 50%; and 51 to 75% immunostained cancer cells. For stromal staining of galectin-3, the same division was made as for galectin-1.

Double-labelling immunofluorescence. For simultaneous visualization of galectin-3 and galectin-1 on the tissue sections, double-label immunofluorescence was performed. Representative tissue sections were chosen with high and low expression of each galectin and sections with the same percentage of positively stained cells. De-paraffinization of 3 μm-thick sections was performed as described above. Briefly, after blocking with donkey and goat normal sera (Dako, Glostrup, Denmark) diluted 1:5 in 10% BSA for 20 min, sections were incubated with the primary antibodies rabbit anti galectin-1 (rabbit anti-human polyclonal antibody against galectin-1, Invitrogen Corporation, Camarillo, CA, US) and rat anti galectin-3 (rat anti-human/mouse monoclonal antibody against galectin-3, clone M3/38, Bioscience Inc., San Diego, CA, USA) diluted 1:150 and 1:200, respectively, in 5% BSA overnight at 4˚C in a wet chamber. After washing in phosphate buffer saline (PBS), slides were incubated with Alexa Fluor® 488 goat anti-rat IgG (A11006, Life Technology, Carlsbad, CA, USA) and Alexa Fluor® 594 goat anti-rabbit IgG (A11037, Life Technology, Carlsbad, CA, USA) secondary antibodies diluted 1:200 in 5% BSA, overnight at 4˚C. Washes were performed with PBS and slides incubated with 4,6-diamidine-2-phenylindolendihydrochoride (DAPI) 100 μg/mL for 15 minutes. Slides were mounted in the glycerol-based Vectashield medium (Vector, Burlingame, CA, USA). Immunostained sections were analyzed by fluorescence microscopy (Leica DMIRE2) with appropriated filters.

In situ RNA labelling. Canine galectin-3 RNA was detected *in situ* using a set of Stellaris RNA fluorescence *in situ* hybridization (FISH) probes (Biosearch Technologies, Novato, CA, USA). The 39 probes were designed using the company's Probe Designer software on the canine galectin-3 transcript sequence (Ensembl assession number ENSCAFG00000015013), and labelled with the CAL Fluor Red 610 fluorophore. Labelling was performed on tissue specimens following the manufacturer's instructions. Formalin-fixed and paraffin-embedded tissue samples were deparaffinised in xylene and rehydrated in a series of alcohols. This was followed by probe hybridization (1 μl from undiluted stock) overnight at 28˚C. The following day, cells were washed with 2× saline sodium citrate (SSC), nuclei were counterstained with DAPI and cover glasses were mounted in Vectashield (Vector) on glass slides.

Statistical analysis. Initially a descriptive study of the data was performed. Then association hypotheses were tested, using the Student *t*-test for continuous variables and Fisher's exact test, Chi-square test and odds likelihood ratio for discrete variables. SPSS software (version 13.0) (SPSS, Chicago, IL, USA) was used for statistical analysis.

Results

Galectin-1 expression in relation to clinicopathological features in primary malignant CMT. In order to assess potential associations between galectin-1 expression and clinicopathological features of malignant CMT, tumour samples were divided into four groups according to galectin-1 expression: fewer than 25%, 25-50%, 51-75%, and more than 75% stained cells. Six malignant CMT specimens (14.6%) expressed galectin-1 in more than 75% of the tumour cells, while only four (9.8%) malignant CMT showed galectin-1 expression in fewer than 25% of the cells. Table I summarizes the expression of galectin-1 in the tumour cells according to clinicopathological features of the tumours. Briefly, no statistically significant association was identified between galectin-1 expression and: tumour histological grade, presence/absence of vessel invasion, lymph node metastases, necrosis, different categories of tumour mode of growth, the presence of squamous metaplasia or development of distant metastases. Furthermore, no relationship was found between galectin-1 staining in the stroma and clinicopathological features of the tumours. However, a significant association was observed between galectin-1 expression in tumour cells and smaller tumour size (p=0.017). Therefore, our results point to an inverse association between galectin-1 expression and size of primary malignant CMT at the time of surgery.

Galectin-3 expression in relation to clinicopathological features in primary malignant CMT. Not having addressed this issue in our previous investigation, clinicopathological parameters of malignant CMT were evaluated by dividing tumour samples into five groups of galectin-3 expression, assessed according to percentage of stained tumour cells (22). Table II summarizes the expression of galectin-3 in tumour cells according to clinicopathological features. Briefly, no statistically significant correlation was identified between galectin-3 expression and: tumour histological grade, lymph node metastases, necrosis, different categories of tumour mode of growth, tumour size, vessel invasion and development of distant metastases.

Galectin-1 and -3 are differentially expressed in normal and neoplastic CMT. In human prostate cancer, galectin-1 and galectin-3 are known to be expressed by different cell sub-populations (27), but such a comparison has not been made in mammary cancer, neither in humans nor in dogs. In order to address this issue, we examined galectin expression in normal mammary specimens from healthy female dogs, 12 benign CMT and 39 primary malignant CMT samples, and normal-adjacent mammary tissues (n=33) in the latter and their metastases (n=10 cases). A distinct pattern was revealed for galectin-3 and galectin-1: In the two samples of normal

Table I. *Galectin-1 expression in malignant canine mammary tumours cells and its relationship with clinicopathological variables.*

		Galectin-1 expression				
Clinical feature	Cases (%)	<25%	25-50%	51-75%	>75%	*p*-Value
Tumour size (n=39)						0.017
<3 cm	26 (66.7%)	1 (33.3%)	5 (50.0%)	15 (75.0%)	5 (83.3%)	
3-5 cm	7 (17.9%)	0 (0.0%)	3 (30.0%)	3 (15.0%)	1 (16.7%)	
>5 cm	6 (15.4%)	2 (66.7%)	2 (20.0%)	2 (10.0%)	0 (0.0%)	
Histological grade (n=38)						0.537
I	10 (25.6%)	1 (25.0%)	2 (20.0%)	5 (25.0%)	2 (40.0%)	
II	19 (48.7%)	2 (50.0%)	6 (60.0%)	10 (50.0%)	1 (20.0%)	
III	10 (25.6%)	1 (25.0%)	2 (20.0%)	5 (25.0%)	2 (40.0%)	
Squamous metaplasia (n=38)						0.401
No	31 (81.6%)	3 (75.0%)	9 (90.0%)	15 (83.3%)	3 (50.0%)	
Yes	7 (18.4%)	1 (25.0%)	1 (10.0%)	3 (16.7%)	3 (50.0%)	
Necrosis (n=39)						0.299
No	8 (20.5%)	2 (50.0%)	1 (10.0%)	3 (15.8%)	2 (33.3%)	
Yes	31 (79.5%)	2 (50.0%)	9 (90.0%)	16 (84.2%)	4 (66.7%)	
Vessel invasion (n=38)						0.235
No	25 (65.8%)	3 (75.0%)	9 (90.0%)	10 (55.6%)	3 (50.0%)	
Yes	13 (34.2%)	1 (25.0%)	1 (10.0%)	8 (44.0%)	3 (50.0%)	
Lymph node metastases (n=33)						0.965
No	23 (6.2%)	2 (6.2%)	6 (6.2%)	12 (6.2%)	3 (6.2%)	
Yes	10 (6.2%)	1 (6.2%)	2 (6.2%)	6 (6.2%)	1 (6.2%)	
Distant metastases (n=39)						0.312
No	29 (74.4%)	4 (100.0%)	5 (55.6%)	16 (80.0%)	4 (66.7%)	
Yes	10 (25.6%)	0 (0.0%)	4 (44.4%)	4 (20.0%)	2 (33.3%)	

Table II. *Galectin-3 expression in malignant canine mammary tumours cells and its relationship with clinicopathological variables.*

		Galectin-3 expression				
Clinical feature	Cases (%)	Negative	<25%	25-50%	50-75%	*p*-Value
Tumour size (n=40)						0.388
<3 cm	25 (64.1%)	3 (12.0%)	7 (28.0%)	12 (48.0%)	3 (12.0%)	
3-5 cm	7 (17.9%)	0 (0.0%)	1 (14.3%)	5 (71.4%)	1 (14.3%)	
>5 cm	7 (17.9%)	0 (0.0%)	4 (57.1%)	3 (42.9%)	0 (0.0%)	
Histological grade (n=39)						0.861
I	10 (25.6%)	1 (33.3%)	4 (30.8%)	4 (21.1%)	1 (25.0%)	
II	19 (48.7%)	2 (66.7%)	5 (38.5%)	10 (52.6%)	2 (50.0%)	
III	10 (25.6%)	0 (0.0%)	4 (30.8%)	5 (26.3%)	1 (25.0%)	
Squamous metaplasia (n=38)						0.087
No	31 (81.6%)	3 (75.0%)	12 (100.0%)	14 (77.8%)	2 (50.0%)	
Yes	7 (18.4%)	1 (25.0%)	0 (0.0%)	4 (22.2%)	2 (50.0%)	
Necrosis (n=39)						0.602
No	9 (23.1%)	2 (50.0%)	2 (15.4%)	4 (22.2%)	1 (25.0%)	
Yes	30 (76.9%)	2 (50.0%)	11 (84.6%)	14 (77.8%)	3 (75.0%)	
Vessel invasion (n=38)						0.057
No	25 (65.8%)	4 (100.0%)	6 (50.0%)	11 (61.1%)	4 (100.0%)	
Yes	13 (34.2%)	0 (0,0%)	6 (50.0%)	7 (38.9%)	0 (0.0%)	
Lymph node metastases (n=33)						0.489
No	23 (69.7%)	1 (50.0%)	7 (63.6%)	14 (77.8%)	1 (50.0%)	
Yes	10 (30.3%)	1 (50.0%)	4 (36.4%)	4 (22.2%)	1 (50.0%)	
Distant metastases (n=39)						0.498
No	29 (74.4%)	3 (75.0%)	8 (61.5%)	15 (78.9%)	3 (100.0%)	
Yes	10 (25.6%)	1 (25.0%)	5 (38.5%)	4 (21.1%)	0 (0.0%)	

mammary glands from non-tumour bearing dogs, when present, galectin-3 was mainly expressed by luminal cells (Figure 1A) while galectin-1 expression was restricted to basal cells, with fewer than 25% of all (both luminal and basal) cells comprising these glands being stained (Figure 1B). Galectin-1 staining in the stroma of normal mammary glands was negligible. In 33 (84.6%) out of the 39 malignant CMT cases, normal adjacent mammary tissue was available for comparative study. Only two cases of normal adjacent glands (6.1%) presented galectin-1 staining of luminal cells; in the remaining 31 (93.9%) cases, these cells were negative for this galectin type, while often being positive for galectin-3. Basal cells, however, mostly stained positive for galectin-1 as observed in normal glands from healthy dogs. In benign CMT, as in normal glands, luminal cells expressed galectin-3 (Figure 1C), while galectin-1 staining was mainly present in basal-like cells from moderately-organized structures (Figure 1D). Together, the proportion of galectin-1-positive cells was less than 50% in all benign cases. We observed a significant up-regulation of galectin-1 expression in malignant CMT ($p<0.05$) when compared to normal glands and to benign CMT, with the majority of primary malignant CMT lesions (n=26 out of 39, 66.7%) presenting galectin-1 staining in more than 50% of tumour cells. Yet, if luminal-like cells were present in such specimens, then those positive for galectin-3 (Figure 1E) exhibited decreased galectin-1 staining (Figure 1F). Galectin-1 expression was not restricted to tumour cells and was also found in stromal cells and the ECM of all malignant CMT cases.

Expressions of galectin-1 and -3 differ and do not overlap in normal and malignant mammary tissues. Simultaneous expression of galectin-1 and -3 was next analyzed by double-labelling immunofluorescence in selected cases. These galectins were expressed by distinct cell sub-populations both in normal adjacent tissue, and benign CMT, as well as in primary malignant CMT. Co-expression of galectin-1 and -3 was not observed in normal tissues. Galectin-1 expression was restricted to the basal compartment, while galectin-3 was mostly found in luminal cells (Figure 2A). In malignant CMT, galectin-1 expression was high. There were nevertheless galectin-3-expressing cells, such as those surrounding necrotic centers. There was little co-expression of galectin-1 and galectin-3 in these cells (Figure 2B). Altogether these findings point to an opposite pattern of expression of galectin-1 and-3 in different cell sub-populations, both in normal and malignant tissue.

An opposite pattern of expression of galectin-3 and -1 was observed in the metastatic process of malignant CMT. In order to attempt to understand the role of galectins during invasion of malignant CMT we compared the expression pattern of galectin-1 compared to that of galectin-3 in cases of animals presenting with metastatic CMT. Differential patterns of expression were also observed in metastatic CMT cell sub-populations. Vessel-invading cells (Figure 3A and B) at the primary tumour periphery and cells in the free lymph flowing subcapsular sinus in lymph nodes overexpressed galectin-3 while presenting decreased galectin-1 expression (Figure 3C and D) when compared to sedentary cells of primary malignant CMT. On the opposite hand, well-established bone (Figure 3E and F) and lung metastases presented decreased galectin-3 expression and overexpressed galectin-1 (Figure 3G and H). Thus, while the expression of galectin-1 and -3 varies depending on the location of malignant cells, an inverse relationship between expression of galectin-1 and galectin-3 is also present in disseminating tumour cell sub-populations of malignant CMT.

Galectin-3 is up-regulated at the mRNA level in intravascular tumour cells of malignant CMT. In order to assess whether increased expression of galectin-3 observed in cell sub-populations such as vessel-invading cells is due to its up-regulation at the mRNA level, we used Stellaris FISH probes. High expression of single molecules of *galectin-3* mRNA was observed in normal adjacent ducts (Figure 4A). Primary malignant CMT however, presented few cells with considerable galectin-3 RNA fluorescent spots. In contrast to primary malignancies, a relatively high expression of *galectin-3* mRNA was found in vessel-invading tumour cells (Figure 4B). This is supportive of increased production of protein expression of galectin-3 by this specific sub-population and not its being an alteration due to uptake from the microenvironment.

Discussion

Galectins are increasingly being implicated as important players in the metastatic process due to their influence on cancer cell–ECM, cancer cell–cell and cancer–endothelial adhesion. Galectin-1 and -3 are two intensely studied members of the galectins family and are both suggested to be closely related to the progression of several types of cancers (4). CMT is the most common cancer in female dogs, representing nearly 50% of all reported neoplasms. Forty-one to 53% of these are malignant, and often fatal due to the development of distant metastases (30). A thorough comparative study of galectin-1 and -3 expression in CMT with emphasis on tumour progression and invasion was thus deemed relevant. In this study, we used a series of spontaneous CMT series in order to investigate the dynamics of galectin-1 and -3 expression in tumour progression and metastasis.

We observed frequent expression of galectin-3 in normal mammary glands but mainly in the luminal compartment; most cells located at the basal layer showed a weak or lack of expression of this type of galectin. Galectin-1 on the other

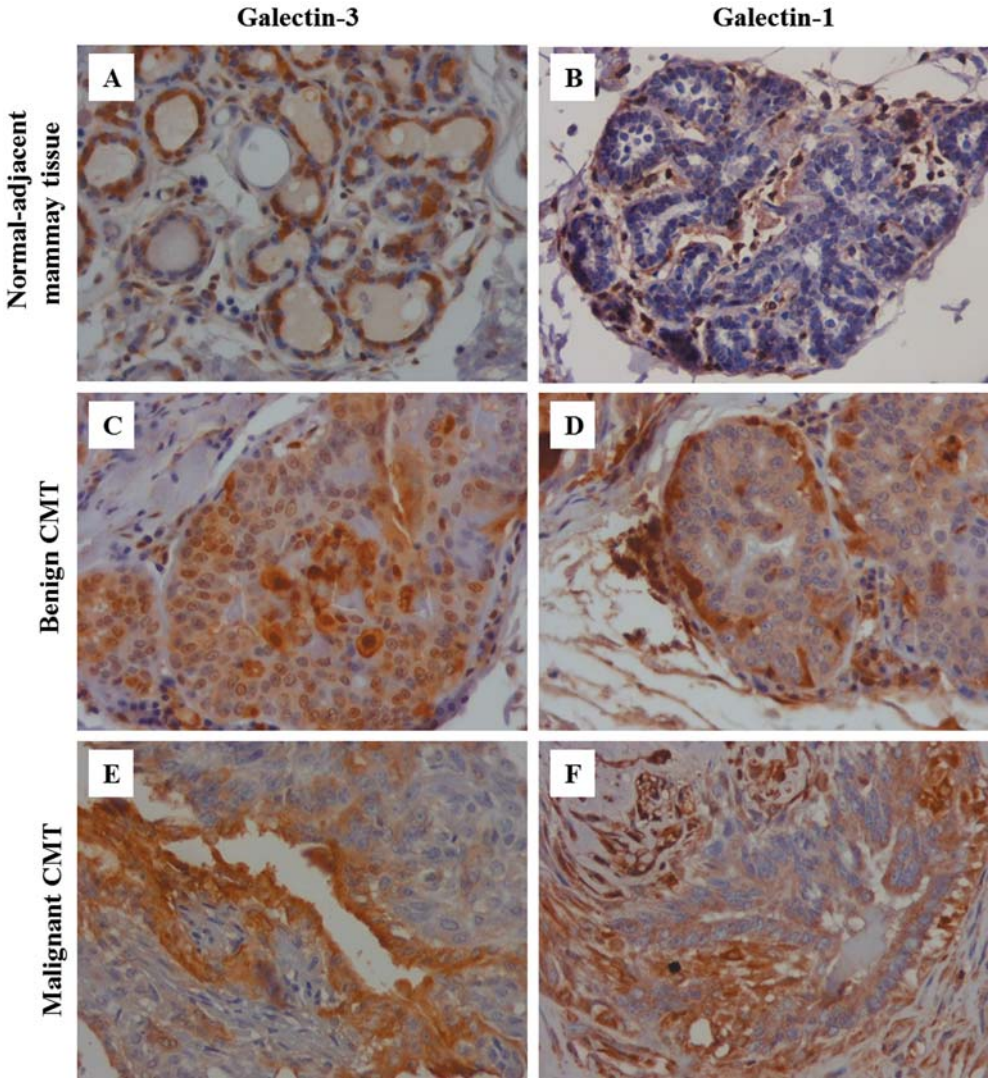

Figure 1. *Different cell subpopulations within normal and neoplastic mammary tissues presented a heterogeneous and divergent expression of galectin-1 and -3. Galectin-1 and -3 immunohistochemical staining was examined in a series of normal mammary tissues, 12 benign and 39 malignant canine mammary tumours, and samples were compared according to the percentage of galectin-expressing tumour cells. Photomicrographs depict galectin-1 and galectin-3 immunostaining (brown color) with haematoxylin counterstain. Normal mammary tissue expressed galectin-3 in luminal cells (A), while being mainly positive for galectin-1 in basal cells (B). Benign CMT presented galectin-3 expression (C) and galectin-1 was expressed in basal cells (D). In malignant CMT, well-differentiated structures expressed galectin-3 in luminal-like cells (E), which presented decreased galectin-1 expression (F) (all ×400 magnification).*

hand was modestly expressed in normal mammary epithelium and confined to a low proportion of cells in the basal compartment of the glands, with a similar pattern of expression observed in benign CMT. However, galectin-1 expression was increased and widespread in malignant CMT, both in tumour cells and in stroma. The expression of both galectins was found not to overlap in normal, benign neoplastic and malignant canine mammary cells. This is in accordance with previous studies where galectin-1 was confined to the basal layer in normal human breast gland tissue, with an increased expression in malignancy, and further suggests its key role in the initiation of transformed tumour phenotype (15, 31). A differential expression of galectin-1 and -3 seems to be an important aspect in the mechanisms of cell adhesion required for invasion in both physiological and pathological events (32, 33). Galectin-1 and -3 were found to be differentially expressed in non-mammary human cancer (26, 27). However, to the best of

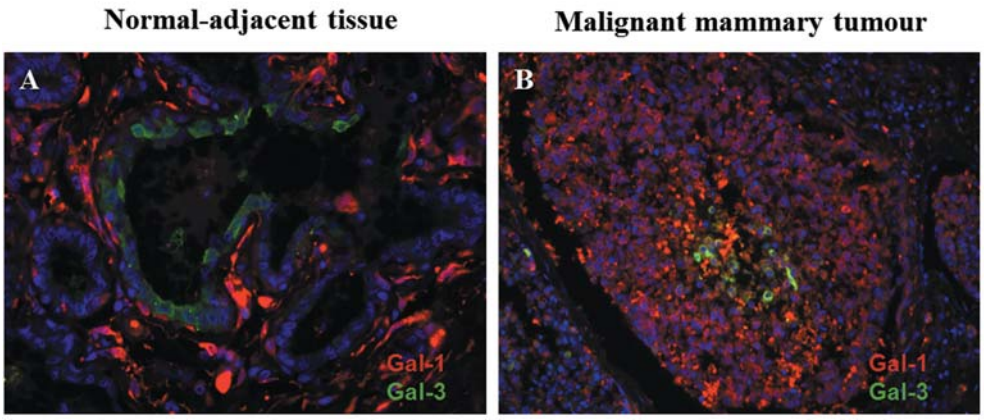

Figure 2. *Galectin-1 and galectin-3 expression did not overlap, neither in normal adjacent nor in malignant canine mammary tumours. Simultaneous expression of galectin-1 and -3 was assessed by double-labelling immunofluorescence using antibodies to galectin-1and galectin-3. Normal mammary tissues presented luminal expression of galectin-3 and basal expression of galectin-1 (A; ×400 magnification). In malignant CMT, different subpopulations expressed galectin-1 and -3 (B; ×200 magnification).*

our knowledge this is the first study in mammary tumours that clearly demonstrates that expression of galectin-1 and -3 is distinct and differs between normal and neoplastic mammary gland tissues.

Differential expression of galectin-3 and galectin-1 was also observed in metastatic CMT cells. In intravascular tumour cells, galectin-1 was found to be down-regulated. A decrease in galectin-1 expression in intravascular tumour cells may confer survival advantage to circulating cells in view of the fact that it acts as a pro-anoikis effector *in vitro* (24). In addition, in the same vessels, galectin-3 was up-regulated. As cytoplasmic galectin-3 is known to be an inhibitor of anoikis (24), it is very likely that this may contribute to cell survival in the bloodstream. Concerning galectin-3 expression at the cell surface of intravascular tumour cells, our recent studies have shown that galectin-3 interacts with the oncofetal Thomsen-Friedenreich (galactose-β-1,3-*N*-acetylgalactosamine T antigen) in vessel-invading cells of malignant CMT (24). Others have shown *in vitro* that MUC1 clustering by galectin-3, *via* T-antigen, induces mucin cell surface polarization and exposure of the smaller cell adhesion molecules which would otherwise be concealed by the much larger and heavily glycosylated MUC1. This might result in increased heterotypic adhesion of the cancer cells to vascular endothelium (34) and increased homotypic aggregation of the cancer cells to form micro-tumour emboli that prolong tumour cell survival in the circulation (35). Survival in the circulation and arrival at distant target organ sites may thus be facilitated by overexpression of galectin-3. Furthermore, decreased galectin-1 will facilitate galectin-3 action since these compete for the same ligands involved in regulating anoikis (36). In the lymph nodes, isolated metastatic tumour cells also expressed high levels of galectin-3 and low levels of galectin-1. On the other hand, distant metastatic lesions presented high galectin-1 and low galectin-3 expression. These findings support a view where galectin-3 plays a role in circulating tumour cells, while galectin-1 is paramount for growth of expanding neoplastic colonies (21).

Further corroborating our initial data, high *galectin-3* mRNA expression was found in normal adjacent tissue and intravascular tumour cells, despite its decreased expression in the primary malignant lesions. Accordingly, in a previous study, *galectin-3* mRNA expression was found to be high in luminal epithelial cells of normal ducts, decreased in carcinoma and re-expressed in peripheral tumour cells in invasive carcinoma (37). However, to the best of our knowledge this is the first study where examination of *galectin-3* mRNA expression revealed elevated levels in intravascular tumour cells themselves.

This study demonstrates that expression of galectins varies with respect to the type of epithelial cell in normal mammary tissues, as well as in benign and malignant primary CMT. Overall, malignant primary CMTs have decreased expression of galectin-3 and overexpression of galectin-1, compared to non-malignant tissues. Yet when considering clinicopathological factors of primary malignant CMT and expression of both galectins, most fail to be associated with either galectin, with only small tumour size being related to relative overexpression of galectin-1. Galectin-1 is a potent pro-angiogenic molecule and as such may be induced early in growth both of primary tumour lesions and metastases in order to allow expansion and facilitate invasion (38). Moreover, most malignant CMTs presented strong galectin-1 expression in stromal cells. Elevated galectin-1 stromal expression has been correlated to poor disease prognosis in both prostate and breast cancer (15, 27).

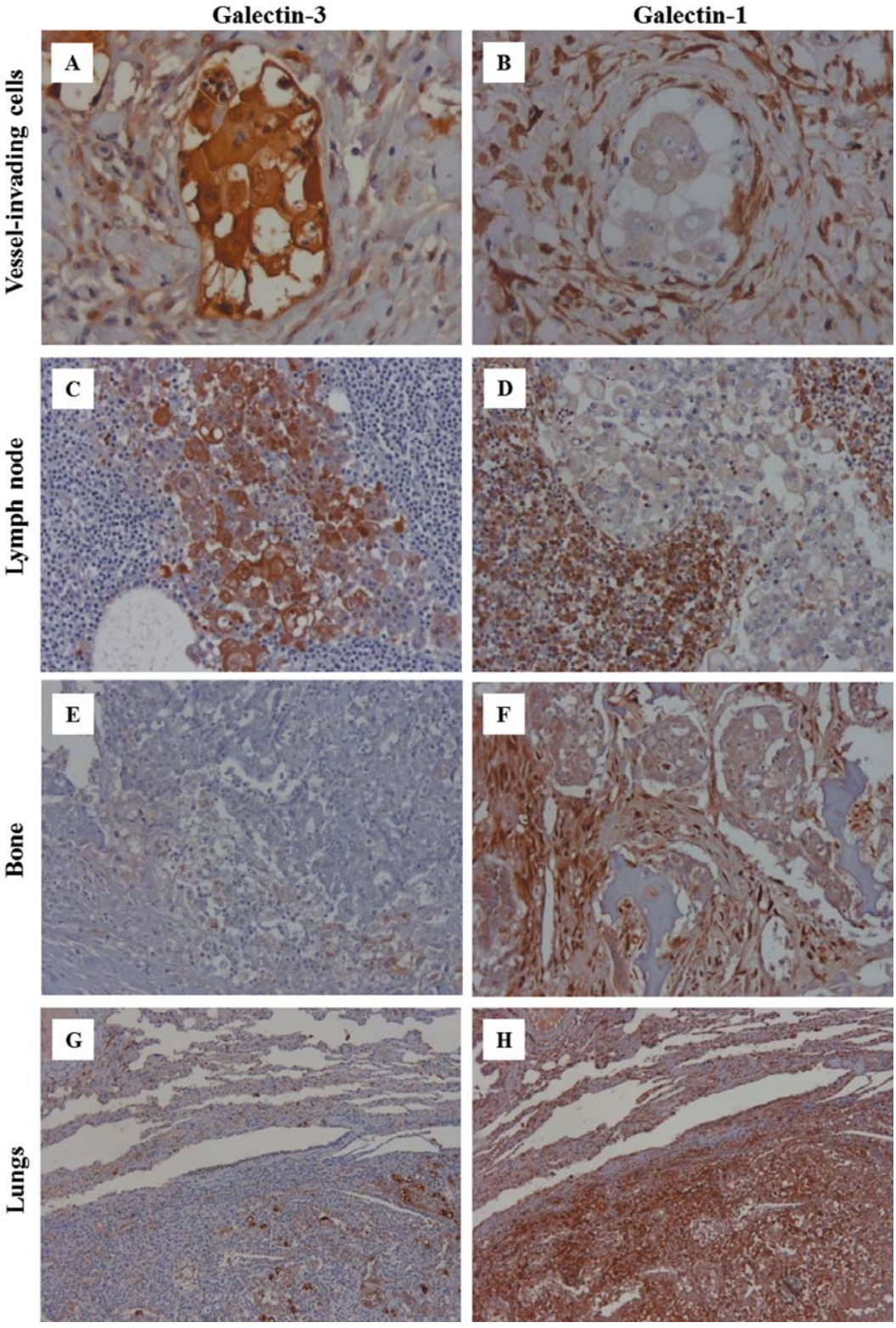

Figure 3. *Dynamic and contrasting expression of galectin-1 and -3 during different phases of the metastatic process. Vessel-invading cells presented galectin-3 expression (A) and a decrease in galectin-1 (B) (×400 magnification). Higher galectin-3 (C) and decreased galectin-1 expression was also observed in invading tumour cells in the subcapular sinus of lymph nodes (D) (×200 magnification). Regarding distant metastases, low expression of galectin-3 and high expression of galectin-1 was observed in bone (E, F) and in lung metastases (G, H) (all ×200 magnification).*

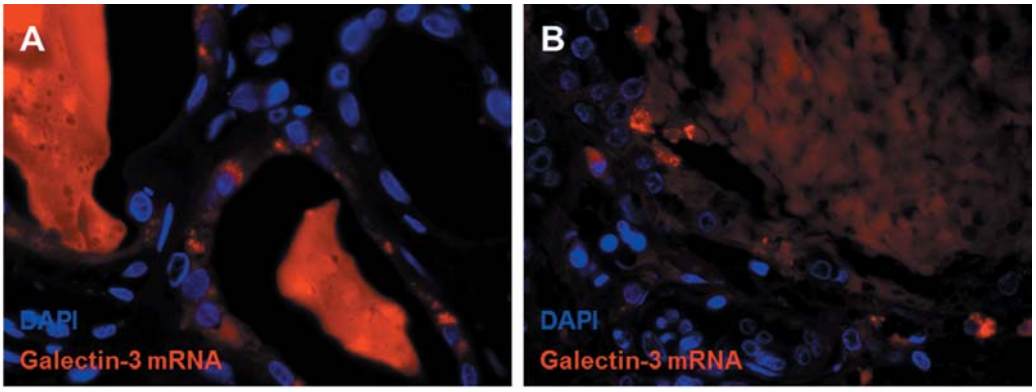

Figure 4. *Tumour cells at the vessel periphery and intravascular tumour cells exhibited elevated galectin-3 mRNA expression. Stellaris fluorescence in situ hybridization probes were used to detect individual mRNA molecules at the cellular level, observed as individual diffraction-limited fluorescent red spots. A: Normal mammary ducts adjacent to canine mammary tumours positive for galectin-3 were used as positive controls and expressed high levels of galectin-3 mRNA. B: Although no appreciable spots were observed in most tumour areas of primary malignant CMT, these were observed in tumour cells surrounding vessels and in intravascular tumour cells (×630 magnification).*

Interestingly, *in vitro*, a lower level of adhesion between tumour cells and the ECM has been observed in the presence of galectin-1 compared to galectin-3 (39). Hence, decreased galectin-3 and increased galectin-1 expression in most cells of malignant CMT might contribute to enhanced migratory capacity of only a few invasive cell subpopulations. However, within the group of primary malignant CMT, on average neither galectin-1 nor galectin-3 expression has value in determining prognosis, based upon a lack of a relationship with known prognosticators.

Being differentially expressed by most tumour cell subpopulations in CMT, the fact that tumour emboli also presented an inverse pattern of expression for galectin-1 and galectin-3, with a stronger expression of the latter, led us to consider hypotheses on the mechanisms underlying this difference in the regulation of galectin expression at such a crucial step in the metastatic process. At least three hypotheses could be envisioned: i) galectin expression could be related to mechanisms of EMT; ii) stress-related factors might elicit a cellular response involving these galectins; and, iii) dynamic changes in galectin expression during adhesion and migration through endothelial cells could occur in tumour cells (39-41). The mechanisms steering such differential expression warrant future studies.

In conclusion, these observations point to the likelihood, that average expression levels do not determine the level of malignancy in primary malignant CMT. Rather, fine-tuning at sites where steps towards metastasis are taken may be more relevant, such as at the invasion front. The contrast observed between micrometastatic aggregates within blood vessels, compared to the primary malignant CMT also indicates that dynamics of the functional state (expression) of galectins may be more decisive than average expression levels. Further studies in order to unravel the mechanisms underlying regulation of galectin-1 and -3 during tumour progression are justified.

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