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Chapter I

Galectins – Potential Targets in Canine Mammary Tumour Therapy

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Abstract

Canine mammary tumours (CMT) account for approximately 25-50% of all intact female dog tumours and 40-50% of affected animals develop malignant tumours. Despite CMT relevance, surgery remains the only effective treatment, which unfortunately is in many cases unsuccessful in preventing distant metastases, the main cause of CMT-related death. It is therefore necessary to understand the mechanisms underlying CMT metastasis, so that future therapeutic studies may be better targeted and thus more efficient. The metastatic process involves a series of events mediated by the balance between conflicting pro and anti adhesive forces. It is well-known that reversible steps of homotypic (cell-cell) and heterotypic (cell-ECM) adhesion are mediated in part by interactions between tumour cell surface glycan-receptors and their

glycosylated ligands on other cells and/or within the tumour microenvironment.

Galectins, a family of glycan-binding proteins, have been implicated in tumour progression and metastasis by influencing cell adhesion, angiogenesis, cell proliferation and apoptosis resistance. Galectin-1 and -3, well-studied members of this family, are believed to play opposing roles in several contexts. Our work has shown down-regulation of galectin-3 in primary malignant CMT and a concomitant decrease in galectin-3-binding-sites in the extracellular matrix (ECM). The decrease of galectin-3-binding sites in the tumour stroma was correlated with down-regulation of a β (1-O) galactosyltransferase, GLT25D1, crucial in collagen glycosylation, and concomitant up-regulation of stromal galectin-1. Stromal galectin-1 was accompanied by galectin-1 up-regulation in malignant CMT cells. In contrast, in intravascular tumour cells both anti-anoikis galectin-3 and its binding-sites were up-regulated in the cytoplasm and at the cell-surface while pro-anoikis galectin-1 was down-regulated and confined to the cell peri-nuclear region. Distant metastases however, expressed high levels of galectin-1 whereas galectin-3 was down-regulated. Thus, a role was envisioned for galectin-1 in growth and invasion at primary and distant sites and for galectin-3 in the systemic dissemination of tumour cells in malignant CMT.

Moreover, our studies provided further evidence of the vital importance of glycosylation changes in modulating galectins function during malignant CMT systemic dissemination. Primary malignant CMT exhibited high contents of sialylated glycans compared to normal mammary tissues and removal of the sialic acid exposed galectin-3-binding sites in most tumour areas, although not in intravascular tumour cells which already expressed them. Mucins are very important glycan-carriers, aberrantly glycosylated and frequently sialylated in cancer.

We showed that over-expression of MUC1 mucin is associated to distant metastases occurrence in malignant CMT. Interestingly, in intravascular tumour cells, but not in primary malignant CMT cells, there was physical interaction between galectin-3 and the un-sialylated form of the T antigen (Thomsen Friedenreich antigen), carried by MUC1. This interaction is believed to induce homotypic aggregation thus favouring blood-borne cell survival.

Therefore there is a fine tuning of galectins function, both at their levels of expression, cellular and sub-cellular localization and at the availability of their glycan-ligands, which plays a crucial role in the development of distant metastases in malignant CMT. Galectin-inhibitors have been used with efficacy in several types of human cancer. Hence, galectins are valuable and exciting targets for new selective therapies in CMT.

Introduction

General Concepts

During the last years pet animals life expectancy has been increasingly expanding, especially that of dogs and cats. As the population of aged animals increases so does the incidence of several types of tumours. Mammary gland tumours are the most prevalent in female dogs and have been studied for more than half a century. In spite of this, an effective treatment for this type of malignancy remains to be established.

Epidemiology

Canine mammary tumours (CMT) are the second most common type of canine neoplasia (25-50%), preceded only by skin tumours [1,2]. CMT are indeed the most frequent type of tumours in female dogs accounting for approximately 52% of all their tumours, up to approximately half of which being malignant [3,4]. Although CMT is a common condition of which the incidence is close to 205/100000 dogs per year [5], its incidence seems to be declining, due to the increased practice of ovariohysterectomy [4]. Indeed, in areas such as Southern Europe countries where ovariohisterectomy is less routinely performed the observed CMT incidence is somewhat higher [6]. Overall lifetime risk of CMT development in intact female dogs is up to 25% [7]. Regarding breed predisposition, Poodles, Cocker spaniels, Fox and Boston Terriers and Pointers appear to be at higher risk while mixed breed dogs reportedly have a lower incidence of CMT, resembling many other conditions with a possible strong genetic background [8,9].

No significant geographically related differences have been observed regarding CMT incidence [10,11,12]. Although it may appear at any age, CMT affects mainly elderly females, being uncommonly diagnosed before 2 to 4 years of age and presenting a higher incidence between 10 and 11 years [4,8].

Etiology

There are no clear and consensual risk factors for development of CMT. Female dogs presenting with pre-malignant CMT lesions are at increased risk of developing a malignant CMT later on in life [13,14]. Pseudopregnancy, gestation and giving birth at first stage, although debated, have not been associated to increased CMT development risk [4,15,16]. The practice of ovariohysterectomy, however, has been proved to confer a protective effect against the development of these tumours [12,17,18]. Despite that, there seems to be an age-related limit until which this effect can be observed. When the procedure is performed relatively late (around 4 years of age) the incidence resembles that observed for intact animals. On the other hand, in animals which underwent ovariohysterectomy before the first oestrous the incidence is drastically reduced with the risk of CMT development being estimated at 0.5%, increasing to 8% in animals spayed after the first oestrous and to 26% of the risk seen in intact animals, when the procedure is performed after the second oestrous [17,19]. Exogenous administration of progesterone derivatives for oestrus prevention has been associated to a greater risk of CMT development [20,21].

Concerning lifestyle, increased weight has been shown to be a risk factor to CMT development [22,23,24]. Diet, (high intake of red meat and low intake of chicken) seems also to be associated to increased risk of developing CMT [24].

Genetic factors have also been involved in CMT occurrence. BRCA1 and BRCA2 genes, of which inherited mutations confer a 56-84% lifetime risk of breast cancer development [25,26], appear to be related to CMT development [27]. Although not associated to overall CMT risk, animals carrying the COMT G482A polymorphism variant allele have a threefold likelihood of developing mammary tumors after 9 years of age when compared to non-carriers [28]. No other studied risk factors have been proven to be related to CMT occurrence.

Physiopathology

The hormonal microenvironment, where progestagens are known to favour epithelial and myoepithelial hyperplasia inducing lobulo-alveolar development while estradiol stimulates ductal growth consensually influences the development of CMT [3,29,30]. As such, repeated oestrus cycles gradually

prime the mammary gland for CMT development [15]. Malignant CMT appear to progress from benign CMT [31]. Despite the fact that both normal and neoplastic glands showing ER (estrogen receptor) and PR (progestagen receptor) expression [32,33], the levels of expression seem to vary. Benign CMT present higher ER expression when compared to their malignant counterparts [32,34], and higher PR expression levels are also observed in benign when compared to malignant CMT [34].

Even though 4 or 6 pairs may be observed, most female dogs present five pairs of mammary glands. However, approximately 65 to 70% of all CMT develop in the caudal abdominal and inguinal ones [3]. The reason for this preferential localization site for CMT development is still unknown but may be related to their increased volume and proliferative alterations in response to the local hormonal microenvironment [3,30]. It has been suggested that caudal glands maintain secretory activity longer than cranial ones being therefore more prone to CMT development [12]. Further corroborating an association between microenvironment and CMT occurrence, 50-70% of affected animals present more than one nodule, of different histological types in distinct mammary glands [12,17,35]. Further evidence for a multifocal origin comes from the observation of different stem lines recognized by a different DNA index as measured by flow cytometry [36] However the possibility of malignant tumour cells being seeded in different glands by lymph vessels [37] or by way of small veins which cross the midline together with craniocaudal anastomoses cannot be ruled out [38]. Moreover, in this regard, neoplastic glands present more lymphatic anastomoses when compared to normal ones [39].

Metastasis

Metastasis, tumour spread from the site of a primary growth to distant target organs, is the main cause of cancer-related morbidity and mortality, in female dogs bearing malignant CMT and its prevention and or treatment after development remain unquestionably the major clinical challenge associated with this malignancy.

CMT metastasis occurs *via* lymphatic vessels or *via* blood vessels, the first being the most frequently observed. Since lymph drainage from mammary glands goes to the axillary and inguinal lymph nodes and sometimes prescapulary nodes, these should be systematically assessed for the presence

of metastases. The lungs are the most frequent target organs despite others such as liver, bone, brain, etc. have also been reported [35].

The development of metastases is a complex process engaging many steps [40]. During progression tumour cells which are able to detach from primary tumour masses and travel to distant sites may succeed in founding new implants, depending on a complex series of coordinated events [40,41]. The tumour microenvironment plays a major influence on the behaviour of tumour cells which, under particular stimuli, such as hypoxia, tend to cross the tissue underlying the malignant growth in order to expand and invade new sites. Moreover, the same *de novo* formed vessels, by angiogenesis due to hypoxia, will not only support the primary growth but also provide an escape route for invading cells which through intravasation will access the circulation. A metastasis favourable microenvironment is, both at primary and secondary sites, dependent on the active crosstalk between tumour stroma, which includes inflammatory and endothelial cells as well as fibroblasts and the extracellular matrix, and tumour cells themselves [42]. Data from CMT specimens and malignant CMT cell lines have pointed to a fundamental role of galectins 1 and 3 and their ligands in the process of vessel invasion and blood-borne survival as well as primary and secondary site tumour growth [43,44,45].

Diagnosis

Pain and /or discomfort are not typical symptoms of CMT and most early malignant CMT are asymptomatic. Hence CMT are often first felt by owners or clinicians during routine check-ups and most dogs appear healthy at time of presentation [12]. The general approach to evaluation of CMT includes complete patient history, physical examination, and complementary diagnostic means, such as thoracic radiographs, abdominal ultrasound and blood examination. . Important history data include age, spaying status, age at ovariohisterectomy if spayed, reproductive cycles, lactation and progesterone administration. Elderly intact females which have undergone multiple oestrus cycles or which have been administered progestagens in order to avoid them are more likely to present CMT. During physical examination a thorough exam of both mammary gland chains and regional lymph nodes, which may be felt when enlarged, must be performed [12,35]. Needle cytology may aid in differentiating other causes of mammary masses from mammary neoplasia and in identifying non-mammary gland derived tumours. Improved cytology

specimen collection of the lesions with at least 4 samples taken *per* mass increases the accuracy of the diagnosis and agreement with later histopathological analysis [46,47].

Non-imaging approach include haematological and biochemical profiles which although not presenting specific alterations in CMT are important to identify concurrent geriatric diseases, paraneoplastic syndromes, coagulation disorders, common in advanced stages of this condition, and to assess the anaesthetic risk before surgery [48].

Imaging approaches are crucial in order to assess the presence of distant metastases and include thoracic radiographs, CT (computerized tomography) [49] and abdominal ultrasound [12].

Animals with metastatic disease may show systemic symptoms, the severity of which is dependent on both the amount and location of metastases. Symptoms include lethargy, weight loss, cough, dyspnea, lymphoedema, fatigue and lameness [35].

Differential Diagnosis

Mammary masses may be broadly classified as benign or malignant. Common benign mammary masses include hyperplasia and abscesses. Pain not being characteristic of CMT, when present, may suggest the alternative diagnosis of an inflammatory process. Ultrasound may reveal cystic structures which can be simple or complex. An image with uneasily defined walls and a hypoecoic centre should raise the suspicion of an abscess which may be confirmed as mentioned by cytology. Definite CMT diagnosis is based on histopathological assessment, which remains the gold standard method for the diagnosis of this condition.

Histological Types and Grading

Several different systems have been used to classify the CMT lesions in an attempt to predict their clinical behaviour. The importance of the WHO classification for mammary tumours in for domestic animals is underlined by its frequent adoption.

Table 1 summarizes the different types of malignant CMT described [50]. Nevertheless, the histological classification of CMT used in Veterinary Medicine is not completely uniform and lacks molecular basis which could

contribute to better assessment of both prognosis and treatment options. Grading of CMT has also been performed using different methods, being the modified Elston and Ellis method, frequently applied [51,52,53]. A new classification of CMT resembling the sub-grouping used in human breast cancer into Luminal A and B, HER+, Basal-like and non classified or Normal-like, based on the expression of markers such as ER, PR,HER2,CK5 has been attempted. The objective is to improve knowledge of metastatic potential and thus prognosis accuracy as well as to implement specific targeted therapy, however there is controversy regarding its usefulness [32,54].

Table 1. WHO histological classification for malignant CMT (adapted from Misdorp et al., 1999)

Malignant CMT	
In situ carcinoma	
Complex carcinoma	
Simple carcinoma	
	Tubulopapillary carcinoma
	Solid carcinoma
	Anaplastic carcinoma
Special types of carcinomas	
	Spindle cell carcinomas
	Squamous cell carcinoma
	Mucinous carcinoma
	Lipid-rich carcinoma
Carcinosarcoma	
Carcinosarcoma or sarcoma in benign tumour	
Sarcoma	
	Fibrosarcoma
	Osteosarcoma
	Other sarcomas

Staging

In addition to the above mentioned histological classification, the WHO also suggests a TNM (tumour-node-metastases) staging method which attempts to provide more clinically useful prognostic information [3] and allows better comparison beyond tumour burden, which may be essential when

evaluating the efficacy of new treatments. The staging system is described in table 2. It is noteworthy that the higher the stage, the poorer the prognosis. Yet, the need to arrive at a better staging system has been stressed recently [55].

Treatment

Despite its frequently aggressive behaviour and distant metastases development, prognosis assessment and treatment for CMT are often inadequate.

Table 2. Modified TNM staging of malignant CMT

Stage	PrimaryTumor	Regional LN Status	Distant Metastases
I	T1	NO	MO
II	T2	NO	MO
III	T3	NO	MO
IV	Any T	N1	MO
V	Any T	Any N	M1

T1 < 3 cm maximum diameter.
T2 3-5 cm maximum diameter.
T3 > 5 cm maximum diameter.
NO Histologically no metastasis.
N1 Histologically observed metastasis.
MO No distant metastases.
M1 Detection of distant metastases.

Surgery

Surgery is considered primary treatment for malignant CMT. Many patients with early-stage malignant CMT may be cured with surgery alone provided that the margins are clean and the tumour has not already invaded [48]. However this is not always the case and approximately 50% of the animals already have either micrometastases or well-established metastases at the time of surgery [35]. Several different surgical techniques were described; nevertheless, radical removal of the ipsilateral chain has been recently advised due to a 58% probability of new homolateral CMT development observed in a prospective study, using a more conservative technique [56]. Routine removal

of the lymph nodes is advisable and often performed on the superficial inguinal nodes however, due to close proximity to the brachial plexus, the proper axillary lymph nodes are not commonly resected [35]. It is a well-established fact that cure probability and general success of oncologic surgery is highly dependent on surgical volume and surgeon experience [57]. Although the vast majority of dogs affected by mammary tumours are subjected to mastectomy at a potentially curable stage, effective post-operative protocols aiming to prevent and /or delay metastases development have not yet been devised. This stems mainly from lack of knowledge in the field of CMT pathophysiology which in turn precludes the use of its involved molecules as dependable cancer typing and staging markers.

Research in CMT Adjuvant Therapy

Adjuvant treatment of human breast cancer is designed to treat micrometastatic disease, or human breast cancer cells that have escaped the primary tumour site and regional lymph nodes but which have still not given rise to clinically detectable metastases. Over the last 2 decades, research on human breast cancer has provided overwhelming knowledge of the disease, ensuing more efficient and less morbid treatments. Adjuvant therapy has been estimated to reduce up to 72% the mortality rate of human breast cancer [58]. Reference! However in malignant CMT, improvements in prevention of recurrence by post-operative adjuvant therapies have been poor.

Clinical trials, attempting adjuvant chemotherapeutic protocols for malignant CMT have shown to be mostly uneffective. Antimetabolites such as 5-fluorouracil (5FU), gemcitabine and methrotexate have been used in combination regimens for breast cancer. Gemcitabine targets the ribonucleotide reductase and as fluorouracil leads to tumour cell apoptosis. In a recent study, dogs treated with surgery alone or surgery followed by gemcitabine showed no significant differences in time to local recurrence, time to distant metastases and overall survival (OS) [59]. 5FU is a pyrimidine analogue which inhibits thymidylate synthase. Real alkylating agents, such as cyclophosphamide, attach an alkyl group to DNA of fast proliferating cells, thereby being cytotoxic. Indeed Yamashita et al indicated effects of such compounds in experimental settings of xenografted CMT in mice [60], and benefit of adjuvant therapy combining 5FU with cyclophosphamide was reported in a small series of malignant CMT [61]. However, more accurate

studies are warranted in order to fully state the benefit of using this combination in CMT therapy.

Other agents such as cisplatin and carboplatin induce DNA damage and are thus sometimes referred as alkylating-like. Carboplatin is used in breast cancer combined therapy, while cisplatin is not used routinely for breast cancer treatment, but has been recently suggested to induce an effective response in a subset of patients with Triple-Negative Breast Cancer [62].

Taxanes block cell division by preventing microtubule formation. Despite a few conflicting studies, taxanes are among the most active and commonly used agents in human early-breast cancer therapy, with both doxetacel and paclitaxel being applied [63,64]. However, in a small prospective study the combination of doxetacel and cyclosporin A did not induce responses in malignant CMT [65], while another study assessing the efficacy and toxicity of paclitaxel noticed only 20% partial responses with high toxicity reported [66]. Docetaxel, another taxane, also failed to demonstrate efficacy in CMT [67]. Thus, the use of taxanes alone in malignant CMT treatment does not seem effective.

Anthracycline-based regimens have been used in the treatment of early-stage breast cancer for decades. Anthracyclines such as epirubicin and doxorubicin inhibit topoisomerase IIa (TOP2A), and are thus particularly useful in cases presenting with TOP2A and HER2 amplification [68]. Doxorubicin has been included in several protocols of adjuvant therapy for human breast cancer, including stage III and locally advanced breast cancer [69]. However, doxorubicin [67] failed to demonstrate efficacy in CMT.

The common expression of drug resistance proteins in CMT may provide an explanation for the relative poor efficacy of cytostatics [70].

Regarding adjuvant hormone therapy, tamoxifen is a selective ER modulator (SERM) which inhibits ER signalling in the mammary gland. It has been approved for human breast cancer treatment since the early 1980's and was shown to reduce the relative risk of distant, ipsilateral and contralateral breast cancer recurrence by up to 50% in tumours with high ER expression [71]. *In vitro* studies have shown CMT cells to be sensitive to tamoxifen [72]. Nonetheless, its oestrogenic side effects impair tamoxifen use in clinical trials in dogs [73]. Thus, as an alternative ovariohysterectomy could be an option for reducing hormonal influence in CMT spreading. Nevertheless, studies have not shown consensual effects of pre-, peri- or post-operative spaying on the progression of malignant CMT [74-77]. A study limited only to hormonal receptor positive tumours would be of better use to study the benefits of hormonal withdrawal in such CMT cases. Aglepristone, a progesterone

antagonist, treatment has also been recently studied and showed a potential to decrease the proliferation index of PR-positive CMT in a small number of animals although no association to disease progression was evaluated [78]. Regarding other hormonal therapies, a study in a small number of animals yielded promising results with a relapse-free survival of 88% at 2 years in animals treated with goserelin, a LHRH agonist [79]. Based on a probable microvasculature tightening effect, pre- and post- surgical intravenous administration of desmopressin has shown to inhibit lymph node and lung metastases and significantly increase DFS and OS in females dogs bearing CMT [80,81]. However, due to the low number of animals used in these studies larger scaled ones are warranted. The present situation leads to the absence of meaningful clinical trials and hence established adjuvant therapies.

In targeted chemotherapy for human metastatic breast cancer, besides the above described agents, vinorelbine a vinca alkaloid has been more recently advocated with overall response rates of 35-45%. In a dosage-finding study, 2 dogs with metastatic bronchoalveolar carcinoma experienced a partial response for an overall response rate of 12.5% in 16 dogs with gross measurable disease [82]. In a phase II clinical trial in dogs with cutaneous mast cell tumors vinorelbine was associated with an overall response rate of 13% [83]. No study has been so far performed with vinorelbine in dogs bearing CMT. An ongoing phase III trial is currently evaluating the role of zoledronic acid in the adjuvant therapy of women with stage II/III breast cancer [84]. Despite having been reported its possible usefulness in canine osteosarcoma [85], no study has been so far performed regarding its efficacy against CMT.

Present knowledge of carcinogenesis and metastases shows great similarity between human and canine mammary cancer. The widely differing clinical results obtained in malignant CMT trials with chemotherapeutic agents, compared to humans, can thus be most probably due to insufficient patient number for correct trial design. But above all it seems that only a parallel subtyping and respective comparison will elicit dependable scientific evidence. In Veterinary Oncology both *in vitro* and *in vivo* studies should take into account molecule expression patterns of the tumours, allowing the sub grouping of animals by tumour type and also considering prognostic and predictive factors. This would aid in better understanding therapy response in clinical trials and substantiate the use of combined targeted therapies.

Prognosis

Prognostic factors may be divided into clinical and histopathological factors.

Clinical Factors

Increased tumour size or volume are reported to be related to lower survival and shorter disease-free interval (DFI) by most studies [86-88]. Ulceration was significantly associated to survival and shorter DFI [86,89,90]. The presence of multiple malignant CMT was significantly associated to DFI [86,90]. Tumour fixation to underlying tissue was significantly associated to lower survival [88]. Presence of lymph node metastases have been significantly associated to lower survival and shorter DFI [86,88,89]. Distant metastases are as expected significantly related to poorer survival [76,86]. Surgical technique was also found to influence prognosis since incomplete excision as assessed by the status of the surgical margin is significantly related to lower survival [23]. The animal reproductive status, early age at first estrous was associated with higher disease-free survival (DFS) [86,90]. On the other hand, short estrous duration and decreased number of oestrus cycles were associated to lower survival and shorter DFI [86]. Dogs neutered at the time of surgery were more likely to survive 2 years after surgery than dogs which were subjected to the procedure [87]. However, negative effects of ovariectomy overall on survival in dogs with CMT reported by others [74,75,77] suggest that since most metastatic tumours are negative to steroid receptors [32], a positive effect of spaying would be restricted to animals bearing receptor positive tumours. Regarding age, while other conflicting studies exist, older age was significantly associated to poorer survival and DFI [86,89,90]. Obesity was found not to influence survival of dogs bearing CMT [77]. Interestingly, malignant CMT-bearing dogs fed with low fat diet and high protein content presented a significantly increased median survival time [23].

Histological Factors

Factors such as histological type have also been associated to prognosis. Although uncommon in dogs, sarcomas (11,2%) are significantly associated to poorer survival [87,89,91]. Carcinosarcomas have also been associated to poor survival when compared to non-mixed malignant tumours [74]. Within carcinomas, the complex type seems to be associated to better survival than the simple one [74,91]. Histological invasion of the surrounding stroma has

been associated to poorer survival [23,91] as has vascular invasion which was significantly associated to decreased survival at 12 and 24 months after surgery [92]. Poorly differentiated tumours (grade III) have a 7 fold increased risk of death 24 months after surgery when compared to moderately differentiated ones (grade II), this rises to a 21 fold increase when compared to grade II and welI-differentiated tumours (grade I) [93]. Tumours with mitotic indexes above 3.09 were significantly associated to poor survival both at 12 and 24 months after surgery [92]. ER- and/or PR-positive tumours were suggested to present a better prognosis, possibly because of an association with a more common complex type [94]. DNA ploidy and S-phase rate were significantly related to increased relative hazards [89]. Altogether, these may be important features in deciding on a course of treatment for CMT when available.

New Prognostic Assessment

Several important attempts have been done in order to establish new prognostic markers by molecular studies such as: detection of circulating tumor cells (CTC) which had a proven prognostic value for human breast cancer [95]; refined search for micrometastases presence using immunohistochemistry [55]; p53 gene mutations which appear to be associated to CMT-related death [96]; 2 recognized single nucleotide polymorphisms in the Catechol-O-methyltransferase (COMT) gene (COMTG216A and COMTG482A) which when simultaneously present are associated to local CMT recurrence [97]; tumour proliferative rates, assessed with MIB-1 labelling indices which have been associated to metastases occurrence [98], DFS [86] and overall survival (OS) [92]; Caveolin-1-positive CMT which strongly correlated to shorter OS [99]; Cox-2 overexpression which was associated to shorter survival time [100]; E-cadherin and beta-catenin reduced expression which were significantly associated with shorter OS and DFS [101] as well as other factors of poor prognosis [102]; MUC1 mucin over-expression which is significantly associated to distant metastases occurrence [52]; TIMP-2 of which increased expression was significantly associated to development of distant metastases, lower OS and lower DFS [103]; and uPA of which high stromal expression was significantly associated with development of distant metastases, lower OS and DFS [104].

These factors might be evaluated as valuable parameters which can be associated to classical prognostic features in order to aid the clinical decision for treatment in the manner of Human Medicine's ongoing approach.

Future Perspectives

As mentioned above, efforts have been done concerning adjuvant therapeutic clinical trials in malignant CMT [59,60,67] but yielded conflicting results, mostly with no significant influence in the outcome of affected animals. Hence, the search for effective post-operative adjuvant chemotherapeutic protocols for CMT remains an exciting field in veterinary oncology research. Consequently, it is crucial to better understand the pathophysiological mechanisms underlying the aggressive capacity of CMT.

In this regard, recently, a family of animal lectins, galectins, has been reported to play paramount functions in different steps of the metastatic process in malignant CMT. They could thus be of potential therapeutic use in combined adjuvant therapies [43]. Galectin-1 and -3, the most studied galectins, are involved in normal cell adhesion, migration and tissue remodeling during mammal development and regeneration processes [105]. Galectins display a high degree of redundancy in their functions and the elimination of a specific galectin from a model system has not proved to severely affect it [105]. Galectin-1 knock-out mice display only defects in olfactory axon pathfinding [106] while galectin-3 knock-outs present defects in neutrophil accumulation during inflammation [107]. Therefore, galectin-inhibitors able to act upon several members of this family would most likely be effective to impair metastatic tumour spread. Galectin-3 natural ligands such as galactose, lactose, polylactosamine and N-acetyllactosamine (LacNac) were identified [108] and are considered as natural inhibitors [109-111]. Pectins, from various dietary sources have also shown enhanced galectin inhibition related to higher arabinose and galactose contents suggesting the importance of arabinogalactans in inhibiting galectin-3-mediated cellular interactions [112]. These data rapidly lead to the enthusiastic exploration of galectins as therapeutic targets in cancer. Several studies pointed to anti-liver metastases activity of arabinogalactans [113,114] and anti-proliferative and pro-apoptotic actions of okra pectin on melanoma cells through galectin-3 interactions [115]. Moreover, many other studies have shown anti-metastatic properties of the galectin-inhibiting Modified Citrus Pectin (MCP). Furthermore, high-affinity carbohydrate-based galectin-3 inhibitors have been developed through the production of LacNac [110] and galactose [116] derivatives. Galectin-3 synthetic inhibitor lactulosyl-L-leucine (Lac-L-Leu) reduced the number of established breast cancer cells (MDA-MB-435Lung2) pulmonary metastases and Lac-L-Leu/paclitaxel combination decreased both the number and the incidence of pulmonary metastases [117]. As an alternative to sugar based inhibitors, artificial peptide inhibitors have also been used and

found to be effective in preventing binding of several galectins [118]. These peptide based inhibitors were found to inhibit metastasis-associated cell adhesion [119]. In order to better understand where and how galectin-innhibitors could impair tumour cells as they go through the malignant CMT metastatic cascade, critical rate-limiting steps in the metastatic process mediated by β-galactoside-galectin interactions and reports of their inhibition in other cancer types will be discussed bellow.

One of the determining factors in tumour progression is the immunologic system. On the one hand, immune-surveillance mechanisms reduce tumour incidence [120]. On the other hand, immune-related mechanisms have been found to promote tumour progression [121]. Galectins were found to be expressed by several immune cells in CMT [43] and are known to regulate their functions [122]. Adding to that, tumour cells themselves release galectins both positively and negatively modulating the immune response. Indeed, the amount of galectin-1 secreted by different tumour cell types is sufficient to induce T cell death when it is released in the ECM [123]. Moreover, galectin-1 was elegantly linked to immune privilege by modulating survival or polarization of effector T cells [124]. Inhibition of galectin-1 gene expression was found to arrest tumour growth, further confirming a growth-promoting activity of endogenous galectin-1 [125]. Furthermore, galectin-1 expression in primary CMT was significantly associated to increased tumour size [45].

Regarding angiogenesis, when compared to vessels in normal tissue, the tumour vasculature is highly disorganized with tortuous and leaky vessels presenting irregular and thin vessel walls [40]. These neovessels not only support primary tumour growth but also provide a route to distant sites since their above described characteristics make them more prone to be invaded than normal vessels [40]. Angiogenesis switch on/off is dependent on pro- and anti-angiogenic molecules which may arise from tumour, endothelial and stromal cells, as well as from the extracellular matrix (ECM) [126]. Tumour-associated hypoxia activates the hypoxia-inducible factor (HIF1alpha) [127] which induces expression of pro-angiogenic factors among which VEGF [128] as well as galectin- 1 and -3 [129]. It is of note that pro-angiogenic galectin-1 is up-regulated in primary malignant CMT [45]. Adding to that, cells surrounding necrotic areas strongly expressed both galectin-1 [45] and -3 [43] in CMT and CMT xenografts, further suggesting a role in response to hypoxia in these tumours. Galectin-3 is a potent angiogenic factor shown to be intimately involved in endothelial cell morphogenesis and angiogenesis. It acts as a chemoattractant for endothelial cells and induces endothelial cell motility, invasion through matrigel and capillary tube formation [130-132]. Moreover,

expression of VEGF was ubiquitously found in normal and malignant canine mammary tissues [53]. Given that galectin-3 is an important mediator of VEGF-mediated angiogenic response [130] and it is found to be expressed by hypoxia-exposed subpopulations in primary malignant CMT [43], this further suggests a role of the lectin in angiogenesis of these tumours. Galectin3-inhibitors impair its angiogenic activity by blocking chemotaxis of human endothelial cells and by inhibiting *in vitro* capillary tube formation by endothelial cells both in a dose-dependent manner [132]. Moreover, galectin-3 pro-angiogenic function was found to be inhibited by MCP in breast cancer xenografts [131]. Thus, it might have the same anti-angiogenic effect in CMT.

Next to the escape from primary sites and vessel intravasation, blood-borne tumour cells need to survive the type of apoptosis associated with the loss of anchorage (anoikis) and a journey through the circulation. Despite a general decrease in galectin-3 expression in primary malignant CMT, interestingly, intravascular tumour cells over-express this galectin [43]. Galectin-3 has been shown to protect tumour cells from anoikis [133,134] by regulating their transition through the cell cycle, inducing a cell cycle arrest at an anoikis-insensitive point (late G1 phase) [133]. Adding to a predominant up-regulation of anti-anoikis galectin-3 at the cell surface and in the cytoplasm of malignant CMT intravascular cells, pro-anoikis galectin-1 was down-regulated when compared to sedentary cells [45]. Interestingly, galectin-3 is an endogenous competitor of the pro-anoikis effector galectin-1 in cancer [135]. Moreover, galectin-3 further contributes to anoikis survival by interacting with tumour-associated antigen, Thomsen-Friedenreich antigen (T antigen) which induces tumour cell homotypic aggregation inside vessels [136]. Notably, vessel-invading cells of malignant CMT also present physical interactions between galectin-3 and the T antigen [44].

After tumour cells lodge in target organ microvessels, they can either proliferate intravascularly, until metastatic tumour outgrows blood vessel and invades distant organ parenchyma [137] or extravasate before initiating a secondary tumour growth. The following rate-limiting step in metastasis is thus dependent on tumour cell arrest in distant organ microvasculature. Galectin-3 mediates metastatic cell adhesion to the endothelium. [138-140]. Further, it appears that galectin-3 interactions with T antigen mediate also the initial adhesion of tumour cells to endothelial cells at the sites of primary attachment to the endothelium, and this is dependent on decreased sialylation of the antigen [141]. In malignant CMT not only expression of galectin-3-binding sites was detected in endothelium but also focal expression of these binding sites was detected in intravascular tumour cells [43] while in sedentary

cells these seem to be masked by sialic acid both in CMT and CMT-U27 xenografts [44]. Several studies converged) demonstrating galectin-3's role in metastases in experimental settings. Both tumour cell-endothelium adhesion and tumour cell homotypic adhesion were shown to be galectin-3 dependent in mouse melanoma and rat prostate cancer cell lines through the lectin's inhibition with MCP [142,143]. Furthermore, a dose-dependent inhibition of breast cancer cells' adhesion to human endothelial cells in vitro was demonstrated using the same galectin-3 inhibitor [131].

The process of extravasation from vessels involves a series of tumour cell interactions with the extracellular matrix (ECM) proteins present on basement membranes and target organ stroma. Malignant CMT displayed an overall loss of galectin-3-binding sites in the ECM. Loss of galectin-3-binding sites was correlated with an overall decrease in galactosylation of the ECM, down-regulation of GLT25D1, a β (1-O) galactosyltransferase that modifies collagen [43]. Moreover, the occupancy of galectin-3-binding sites by other endogenous galectins was also observed with an up-regulation of stromal galectin-1 and to a lesser extent of cleaved galectin-3 [43]. That tumour cell-ECM proteins such as laminin interaction are mediated by galectin-3 was clearly demonstrated through its inhibition by MCP [144]. As galectin-3, galectin-1 is also able to both stimulate and inhibit cellular adhesion through cross-linking glycans on integrins and through binding to laminin impeding integrins' accessibility, respectively [145]. Moreover, galectin-1 increases the adhesion of cancer cell lines to the ECM [146]. In addition, galectin-1 promotes increased cancer cell motility *in vitro* [147]. Galectin-inhibitors, citrus pectin polysaccharides were reported to impair invasion through matrigel of human metastatic breast carcinoma cells, in a dose-dependent manner [148]. Thus, *in vivo* effects of galectin-inhibitors on experimental metastasis of various malignancies may involve inhibition of tumour cell invasion.

Following the initial arrest in distant organs and extravasation, most tumour cells suffer apoptosis induced by several stimuli, and less than 2% survive giving rise to micrometastases [40]. This is therefore, one of the most important rate-limiting steps in the efficiency of the metastatic process. Galectin-3 present in the cytoplasm, regulates tumour cell apoptosis, mainly by acting upon key mitochondrial apoptotic pathways, [149] thus potentially playing a significant role in early tumour cell survival in target organs. On the other hand, nuclear located galectin-3 inhibited metastasis, anchorage independence and promoted apoptosis [150]. Malignant CMT tumours exhibited a statistically significant decrease of nuclear galectin-3 expression

with the majority of the immunostaining confined to the cytoplasm as did distant metastases [43]. This anti-apoptotic function of galectin-3 could be targeted by galectin-inhibitors [149].

In order for micrometastases to evolve into clinically relevant secondary tumours, beyond 1mm, neo vascular supply is crucial. Then chemotaxis to endothelial cells and the development of new blood vessels occurring by angiogenesis constitutes the next vital step. Distant metastases expressed even stronger levels of pro-angiogenic galectin-1 expression when compared to primary malignant CMT [45]. In the same well-established CMT metastatic lesions galectin-3 is over-expressed surrounding necrotic areas [43]. A statistically significant decrease in angiogenesis and spontaneous metastasis of breast cancer xenografts was observed in animals treated with galectin-inhibitors [131]. Therapy targeting angiogenesis is regarded as one of the most promising and important targets in cancer therapy.

Most anti-neoplastic drugs act by tumour cell apoptosis induction *via* the mitochondrial apoptotic pathway [151]. Galectin-3 impairs this pathway, [149] and has been shown to directly regulate the sensitivity of tumour cells to several chemotherapeutic agents such as cisplatin [152], staurosporine [153], etoposide [154], bortezomib [154], dexamethasone, [155] and doxorubicin [156]. Galectin-3 inhibitors might sensitize tumour cells to cytotoxic drugs by inhibiting galectin-3 anti-apoptotic effects. Galectin-3-inhibitors have so far been proven to reverse multiple myeloma cell resistance to bortezomib and to enhance their response to apoptosis induced by dexamethasone [155] and treatment of hemangiosarcoma cells with galectin-3 inhibitor increased their sensitivity to doxorubicin-induced apoptosis [156]. Thus, addition of galectin-3-inhibitors to therapeutic regimens for treating CMT could have the potential to improve the effects of chemotherapy. In addition to enhancing apoptosis induced by cytotoxic drugs, galectin-3 inhibitors may be able themselves to induce apoptosis [152,155] in tumour cells as suggested by multiple myeloma cells in which MCP induced apoptosis through a caspase-8-to-caspase-3 signalling cascade and the effect of several forms of citrus pectin on apoptosis induction in human prostate cancer cells [157].

Galectin-inhibitors were shown to be effective experimentally either *in vitro* or *in vivo*, or both, against prostate carcinoma [157], colon carcinoma [158], breast carcinoma [131], melanoma [142], multiple myeloma [155], and hemangiosarcoma [156]. Moreover, galectin-inhibitors usefulness has been suggested to enhance the effects of doxorubicin, the currently standard of care for dogs bearing hemangiosarcoma [156]. The specific binding between a pectin-derived galactan and recombinant human galectin-3 was demonstrated

supporting the suggested molecular hypothesis for the anticancer action of MCP by demonstrating that bioactive fragments from pectin can bind specifically to galectin-3 [159]. Fluorescently labeled galactan binding to a highly metastatic CMT cell line was evaluated with and without shRNA inhibition of galectin-3 and demonstrated a strongly decreased binding upon galectin-3 silencing (de Oliveira et al. Unpublished data). This points to galectin-3 as the main glycan-receptor responsible for galactan binding to CMT-U27, although not completely excluding binding to other endogenous galectins, and thus supports the use of pectins in metastatic targeting combined therapies in malignant CMT.

Conclusion

In conclusion, increased owner awareness and improving Veterinary care has been leading to earlier diagnosis at stages amenable to complete surgical resection of CMT. Consequently, survival rates to the condition but also late onset metastases are expected to slowly increase. The use of adjuvant therapies that would significantly aid in further ameliorating survival chances of patients with CMT is in its primordium and thus the fundamental interest in understanding CMT progression at the molecular level. Due to galectins' adhesive, angiogenic and apoptosis-related properties, they are involved in multiple CMT critical rate-limiting metastatic steps. By inhibiting galectin-3 functions and enhancing apoptosis induced by cytotoxic drugs, galectin-inhibitors hold the potential to increase dramatically the efficiency of adjuvant chemotherapy in CMT. Galectin-inhibitors were shown to be effective experimentally against several types of cancer. Human clinical studies performed to date demonstrated that galectin-inhibitors significantly increased prostate specific antigen doubling time in patients with recurrent prostate cancer [160] thus confirming its foreseen usefulness in treating spontaneous cancer. Thus, galectin-inhibition is promising in co-adjuvant treatment in CMT as it is in multiple other human and canine malignancies.

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