Prognostic significance of tumour-associated inflammation related markers in canine mammary gland tumours

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Abstract

Canine mammary gland tumours (CMGTs) are a major cause of illness and premature death in female dogs, especially in countries like Sri Lanka where early de-sexing is not a routine veterinary practice. Therefore, there is a need for prognostic markers which can better predict the behaviour of a CMGT. In human breast cancers (HBCs), some markers of tumour-associated inflammation (TAI) have been shown to better predict prognosis than many conventional prognostic markers. This thesis investigated whether TAI prognostic markers adopted from human breast cancers are similarly prognostic for CMGTs.

The prognostic markers investigated in this thesis included tumour stromal mast cell density determined by toluidine blue staining, gene expression of chemokines: CCL5, CXCL12, CXCL10, and chemokine receptors: CXCR3, CXCR4, CXCR7, CCR4, CCR9 and gene expression and immunostaining of two immune checkpoint molecules: programme death ligand-1 (PD-L1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) . Similar to HBCs, all markers except CXCL10 and CCR4 were prognostic of the disease outcome determined by disease-specific survival times of the dogs with mammary neoplasms. Of them, stromal mast cell density, CCL5 gene expression and PD-L1 immunostaining were prognostic independent of tumour size, tumour histological grade, and lympho-vascular invasion observed in histological sections. In conclusion, this thesis identified that similar to HBCs, TAI related prognostic markers are useful to better predict the behaviour of CMGTs while stromal mast cell density has the potential to be adopted for routine laboratory prognostic determination.

In addition to identification of prognostic markers, surveys of CMGTs in Sri Lanka and New Zealand conducted for sample collection gathered large amounts of information that allowed a comparison of CMGTs between the two countries. These studies allowed a determination of the characteristics of dogs with CMGTs, as well as allowing histological characterisation of the tumours within the two countries.

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Abbreviations

CMGTs	Canine mammary gland tumours
OHE	Ovariohysterectomy
BRCA1	Breast cancer associated gene 1
BRCA2	Breast cancer associated gene 2
DFI	Disease-free interval
OS	Overall survival
IHC	Immunohistochemistry
PET	Positron emission tomography
COX-2	Cycloxygenase-2
PR	Progesterone receptor
ERα	Oestrogen-receptor-α
PCNA	Proliferating cell nuclear antigen
HER-2	Human epidermal growth factor-2
PTGS2	Prostaglandin-endoperoxide synthase-2
TME	Tumour microenvironment
TAI	Tumour-associated inflammation
PD-L1	Programme death ligand-1
CTLA-4	Cytotoxic T-lymphocyte antigen-4
VTH	Veterinary teaching hospital
VPC	Veterinary practice Colombo
α-SMA	α-smooth muscle actin
BCS	Body condition score
NZ	New Zealand
MGD	Mammary gland disease
FFPE	Formalin-fixed paraffin-embedded
HE	Haematoxylin and eosin
MCD	Mast cell density
MST	Mean survival time
cDNA	Complementary DNA
NCBI	National centre for biotechnology information
RT-PCR	Real-time polymerase chain reaction
CV	Coefficient of variance
Ct	Cycle threshold
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
PVDF	Polyvinylidene difluoride
TBS	Tris-buffered saline
NFDM	Non-fat dry milk
TBST	Tris-buffered saline with 0.1% tween 20
HPRT	Hypoxanthine-guanine phosphorybosyltransferase
RPL32	Ribosomal protein 32

HRP	Horse-radish peroxidase
HPF	High-power field

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Chapter 1 *: Literature review*

1.1 Introduction to tumour-associated inflammation (TAI) related prognostic markers in canine mammary gland tumours

Mammary gland tumours are the most common neoplasm among intact female dogs and remain a major cause of illness and premature death in dogs around the world where early de-sexing is not routinely advocated and performed.[125] Most studies suggest, 40 – 50% of canine mammary gland tumours (CMGTs) are malignant.[54,2,13,125] The percentage of dogs that develop tumour metastases and subsequently die within two years due to mammary neoplasms ranges from 20 to 44%.[138,54,115,20] Many clinical and tumour related prognostic factors such as tumour size, histological classification, and grade have been proposed to predict the behaviour of CMGTs and some of them are routinely used by veterinary pathologists and clinicians to provide prognostic information.[88,104] However, while these techniques provide some information regarding the likely neoplasm behaviour, it is currently difficult to predict which CMGTs will metastasise.[149] This uncertainty is a limiting factor for implementing new therapeutics for CMGTs.[137] Therefore, there is a need to identify novel prognostic markers to determine the future behaviour of CMGTs more accurately.

Tumours not only consist of tumour cells but also include a tumour stroma which consists of connective tissues, blood-lymph network, and tumour infiltrating immune cells.[157] These stromal components are collectively known as the tumour microenvironment.[157] Many recent human cancer studies suggest the cross-talk between tumour cells and the tumour microenvironment is crucial in determining tumour behaviour, especially the metastatic potential.[157,120] Tumour associated inflammation is the mediator of this cross-talk and its components have been identified as excellent prognostic markers for many human cancers

including human breast carcinomas. Many morphological, clinical, and molecular similarities between CMGTs and human breast cancers have been identified.[112] In view of these similarities, the main aim of this thesis was to determine whether some of the tumour-associated inflammation related markers which were shown to be prognostic in human breast cancer can be used to better predict the disease outcome of CMGTs.

Although mammary neoplasms are reported in dogs in many countries across the world, the incidence of mammary neoplasia is higher in countries where early spaying of female dogs is not a routine practice.[37,12] Therefore, these countries would particularly benefit from the novel prognostic markers expected to be identified in this thesis. Sri Lanka is a South Asian country where spaying of female dogs at an early age is not a common practice.[33] In Sri Lanka, mammary neoplasia is a common cause of disease and mortality of female dogs.[33] Thus, it was initially planned that mammary tumours obtained from dogs in Sri Lanka would be used to identify novel prognostic markers. However, due to the limited post-surgical follow-up data from these dogs, it was necessary to identify outcome-known mammary neoplasms from dogs in New Zealand. During this data collection process, it was recognised that no previous studies have investigated the canine mammary neoplasms present in dogs either in Sri Lanka or New Zealand. Therefore, another aim of this thesis was to describe the clinicopathological features of CMGTs of dogs in Sri Lanka and New Zealand and determine whether any of the clinical features could be useful for clinicians to predict the histological malignant or benign status of these neoplasms.

This chapter will introduce CMGTs, review currently available prognostic markers for CMGTs and compare the clinical and molecular characteristics of CMGTs with human breast cancers. The literature on cross-talk between tumour cells and the tumour microenvironment will then be discussed and currently available prognostic markers of tumour-associated inflammation will be reviewed with an emphasis on markers of this category that have been previously studied for use in human breast cancers or CMGTs. The tumour-associated inflammation related prognostic markers reviewed in this chapter will

include three different types of markers: tumour infiltrating immune cells, chemokines and chemokine receptors, and immune checkpoint molecules.

1.2 Morphology of the canine mammary gland

Dogs have five pairs of mammary glands located bilateral to the ventral abdominal midline.[40] A mammary gland is a circumscribed area of mammary gland tissue which opens into a teat.[40] According to their anatomical location, mammary glands are identified as thoracic, abdominal, or inguinal. The thoracic mammary glands are the most cranial and include cranial and caudal thoracic mammary glands. Caudal to these are the cranial and caudal abdominal mammary glands while the most caudal pair is the inguinal mammary glands.[40,135] The mammary glands are modified sweat glands. The secretory tissue of the mammary gland is composed of lobes, with each containing several lobules. Each lobule is composed of small secretory units called alveoli. The interlobular ducts originate from the alveoli and open into an interlobular lactiferous duct, which opens into a larger channel called the lactiferous sinus. The lactiferous sinus continues into the teat sinus. The papillary duct (teat canal) leads to the teat opening. In the dog, 8-12 papillary ducts per teat open onto the teat surface.[40,9] The stromal tissue of a mammary gland contains nerves, blood, and lymphatic vessels and, in juvenile animals, an abundant amount of adipose tissue.[9,134]

Microscopically, the alveolar epithelium varies between simple cuboidal and columnar depending on the secretory activity at the time.[134] The characteristic star-shaped myopeithelial cells are found between the alveolar basal laminar and the epithelial lining. The contractile nature of the myoepithelial cells facilitates the ejection of milk. The lining epithelium of the ducts varies with the size of the duct. The intra-lobular and small inter-lobular secretory ducts are lined by a simple cuboidal epithelium. The larger ducts and sinuses are lined by a double layer of cuboidal to columnar epithelium. The papillary ducts have stratified keratinised squamous epithelium, which continues to the skin surface.[134]

The canine mammary glands are supplied by multiple arteries, which frequently anastomose.[134] Consequently, a single mammary gland will receive blood through several different arteries. The cranial and caudal thoracic mammary glands are mainly supplied by the cranial superficial epigastric artery. The cranial abdominal mammary glands are also supplied by the cranial superficial epigastric artery whereas the caudal abdominal mammary glands and inguinal mammary glands are mainly supplied by branches of the caudal superficial epigastric artery.[134] The venous outflow from the mammary gland is similar to the arterial supply except that more extensive anastomosis is seen with the craniocaudal veins compared to the arteries.[134] In regards to lymphatic drainage, the thoracic mammary glands are drained by the axillary lymph nodes, while the abdominal mammary glands drain into the inguinal lymph nodes. The inguinal mammary glands are drained by inguinal lymph nodes. Lymph vessels also form ipsilateral and contralateral anastomoses.[107] Interestingly, the venous drainage pattern is often shown to be altered in neoplastic mammary glands with neoplastic glands typically forming more extensive anastomoses than non-neoplastic glands.[107,103]

1.3 Development of canine mammary gland tumours in dogs

The canine mammary gland undergoes extensive proliferation, differentiation, and remodelling throughout the entire reproductive life of female dogs.[127] Each oestrus cycle includes waves of epithelial and mesenchymal proliferation, ductal branching and alveologenesis which are followed by intense regressive changes.[140,84] These activities are coordinated and controlled by oestrogen and progesterone hormone concentrations.[12] However, the continuous cellular changes mediated by reproductive hormones predispose the mammary glands to neoplasia. Therefore, mammary gland tumours are the most common neoplasm among intact female dogs.[12] Ovariohysterectomy (OHE) performed at an early age minimizes the prolonged exposure of mammary tissues to reproductive hormones and thereby reduces the risk of mammary neoplasia.[12] Specifically, the risk of mammary neoplasia is only 0.5% when OHE is performed before the first oestrus while the risk is 8% if the OHE is performed between the first and the second estrus.[12] Consequently,

the incidence of CMGTs is decreasing in regions of the world where OHE is routinely performed at an early age.[2,3,125] However, in many developing countries, including Sri Lanka, where spaying of female dogs at an early age is not a common practice, mammary gland tumours are still the most common tumour among female dogs and one of the major causes of female dog mortality.[125,3]

1.4 Risk factors for canine mammary gland tumours

A risk factor is a character, condition, behaviour or any other factor that increases the likelihood of developing a disease. [81] Advanced age, prolonged exposure to reproductive hormones and breed are generally considered as risk factors for CMGTs.[34,13,140] To a lesser extent, frequent feeding of red meat and obesity may also contribute to the risk of developing mammary neoplasia in dogs.[1]

1.4.1 Age

Mammary gland tumours typically occur in middle-aged or older dogs and are rare in dogs less than 5-years-old.[129,44] The average age of onset is 7-8 years while the incidence decreases after 10 years old[55,13] However, the age of peak incidence of CMGTs has been found to vary among breeds. In general, shorter-lived breeds such as Rottweiler and Saint Bernard typically develop mammary gland tumours earlier in their life, compared to breeds that live longer, such as Maltese terrier and Beagle.[13] Two previous studies have shown that age at diagnosis of malignant CMGTs is significantly higher than age at diagnosis of benign CMGTs.[153,138] However, advanced age at diagnosis was not found to have a significant association with histological-malignancy of CMGTs in another study.[125]

1.4.2 Prolonged exposure to reproductive hormones

As previously stated, prolonged exposure to reproductive hormones is considered a risk factor for CMGTs.[12] Early OHE prevents prolonged exposure to reproductive hormones and consequently reduces the risk of mammary neoplasia.[13] Frequent occurrence of pseudopregnancy, a physical state where all the signs and symptoms of pregnancy are exhibited except presence of a fetus, and low parity are two conditions which have been suggested to promote prolonged exposure of mammary tissues to endogenous reproductive hormones.[18,129,150] Of these conditions, pseudopregnancy has been identified as a risk factor for CMGTs by one previous study.[130] However, the significance of low parity as a risk factor for CMGT development has not yet been confirmed. Exposure of mammary glands to synthetic reproductive hormone preparations have also been shown to increase the risk of mammary neoplasia in dogs.[143] The studies which have investigated the effects of dose, duration and type of exogeneous reproductive hormones on the risk of developing CMGTs have concluded that progestins alone mainly increase the risk of benign tumours, while oestrogen and progestin combinations increase the risk of malignant tumours.[27,49]

1.4.3 Breed

In general, mammary gland tumours are more common in smaller breeds.[159] The small breeds reported to have an increased risk include poodle, chihuahua, dachshund, Yorkshire terrier, Maltese terrier, and cocker spaniel.[159] However, some large breeds such as German shepherd, pointer, doberman, and boxer are also reported to have an increased risk of developing mammary neoplasia.[90,55,35] In addition, purebred dogs are more commonly affected than crossbred dogs. The familial or inherited germ line mutations observed in the canine analogues of human breast cancer 1 (BRCA1) and human breast cancer 2 (BRCA2) genes, have been proposed as contributory factors for reported breed predisposition to CMGTs.[119] Germline mutations in both BRCA1 and BRCA2 were found to associate with a significantly high risk of mammary neoplasia in English springer spaniels but published evidence is lacking for other breeds.[140]

1.4.4 Diet and obesity

A large case control study conducted in the United States of America (USA) in 1991 suggested that obesity at the time of puberty, and a diet high in red meat increases the risk of mammary neoplasia development in dogs.[136] The connection between obesity and increased risk of mammary neoplasia has been explained by two theories.[136,1] According to the first theory, obese dogs have low levels of sex hormone-binding serum globulin which consequently increases free oestrogen in circulation, promoting neoplasia. The other theory suggests that adipose tissues are a source of oestrogen as they possess enzymes necessary for conversion of androgens to oestrogen. Therefore, obese female dogs are more likely to have elevated concentrations of oestrogen predisposing them to mammary neoplasia.[136]

1.5 Histological classification and grading of CMGTs

1.5.1 Histological classification of CMGTs

Canine mammary gland tumours are a highly heterogeneous group of neoplasms.[51] The marked histological diversity observed in mammary gland tumours prompted human and veterinary pathologists to develop histological classification systems to categorise them in a more meaningful way. Subsequently, it was identified that some histological patterns are consistently associated with distinct clinical presentations or disease outcomes. Therefore, histological tumour sub-types are used for prognostic determination in human breast cancers and canine mammary gland tumours.

Two initial classification systems for CMGTs were published in 1974[56] and 1999[89] and a revised system was published in 2011.[51] According to the 2011 classification proposed by Goldschmidt and colleagues, neoplastic, dysplastic or hyperplastic conditions in the canine mammary gland are subdivided into eight basic categories including malignant epithelial tumours, malignant epithelial tumours-special types, malignant mesenchymal tumours,

malignant mixed tumours, benign tumours, hyperplasia and dysplasia, tumours of the nipple and hyperplasia or dysplasia of the nipple (Table 1.1).

Table 1.1 Histological classification of CMGTs

Source: Goldschmidt et al. (2011)

Malignant neoplasms	Benign neoplasms
1. Malignant epithelial neoplasms	5. Benign neoplasms
Simple carcinomas	Adenoma-simple
Carcinoma-in-situ	Intraductal papillary adenoma
Tubular	Ductal adenoma
Tubulopapillary	Fibroadenoma
Cystic papillary	Myoepithelioma
Cribriform	Complex adenoma
Carcinoma-micropapillary invasive	Benign mixed tumour
Carcinoma- solid	
Comedo carcinoma	6. Hyperplasia/dysplasia
Carcinoma-anaplastic	
Ductal carcinoma	7. Neoplasia of the nipple
Intra-ductal papillary carcinoma	
Carcinoma-arising from in a complex adenoma/mixed tumour	8. Hyperplasia/dysplasia of the nipple
Carcinoma-complex type	
Carcinoma and malignant myoepithelioma	
Carcinoma-mixed type	
2. Malignant epithelial neoplasms - Special types	
Squamous cell carcinoma	
Adenosquamous carcinoma	
Mucinous carcinoma	
Lipid rich carcinoma	
Spindle cell carcinoma	
Malignant myoepithelioma	
Squamous cell carcinoma-spindle cell variant	
Carcinoma -spindle cell variant	
Inflammatory carcinoma	
3. Malignant mesenchymal neoplasms	
Osteosarcoma	
Chondrosarcoma	
Fibrosarcoma	
Hemangiosarcoma	
Other sarcomas	
4. Carcinosarcoma - Malignant mixed mammary tumours	

The different histological classification systems used to classify CMGTs in various studies makes it difficult to compare the results between studies. However, in general, epithelial mammary neoplasms were more frequently reported than mesenchymal neoplasms. The most frequently reported malignant epithelial mammary neoplasm is simple carcinoma while simple carcinoma sub-types including tubular carcinoma, tubulopapillary carcinoma and solid carcinoma were reported more frequently than other sub-types. [125,149,54,117,41] In contrast to simple carcinomas, special types of malignant epithelial neoplasms were infrequently reported.[51] Inflammatory carcinoma is a rare, fast growing, special type of epithelial mammary neoplasm which requires clinical findings in addition to histology for proper characterisation.[141] Characteristic clinical presentation of inflammatory carcinoma in dogs include generalised oedema, erythema, and pain in the neoplastic glands. The histological features of canine inflammatory mammary carcinoma include a high-grade carcinoma, often a tubular or solid type, with dermal lymphatic invasion.[141,67] Mixed mammary tumours and fibroadenoma were the most frequently reported benign CMGT histological sub-types.[29,89]

1.5.2 Histological grading of CMGTs

Histological grade is a numerical assessment of the degree of cellular differentiation and proliferation of a neoplasm, which is suggested to reflect the aggressiveness of its clinical behaviour. In general, human breast cancers and CMGT grading systems include three grades.[38] Grade I tumours closely resemble the normal mammary gland in terms of cellular morphology, organisation pattern and proliferative activity. In contrast, the tissue architecture, cellular morphology and proliferation rate of grade II and grade III tumours are markedly different from normal mammary gland.[38] Usually, the clinical behaviour of a grade I tumour is less aggressive compared to a grade III tumour with grade II tumours typically showing clinical behaviour intermediate between low and high grade tumours. Therefore, histological grade is used to predict the disease outcome in human breast cancers and CMGTs.

Currently, veterinary pathologists use a modified method developed from the Elston and Ellis grading method (1991) for human breast cancers widely known as the "Nottingham histological grade" to grade CMGTs.[38] In this method histological features of, tubule formation, nuclear pleomorphism and mitotic counts are assessed and scored on a scale from 1 to 3 (Table 1.2). Although the Elston and Ellis method is still used for grading CMGTs, it is not considered as an ideal method for grading several CMGT sub-types, particularly, mixed mammary carcinoma and complex carcinoma.[38] This is due to some basic histological dissimilarities between human breast cancers and CMGTs. Most breast cancers contain only a neoplastic epithelial cell component whereas CMGTs may include epithelial, myoepithelial and mesenchymal components. Further, neoplastic cells in a human breast cancer mostly exhibit a uniform proliferation pattern whereas in CMGTs, multiple patterns of neoplastic epithelial cell proliferation are often observed in a single tumour. In addition, unlike in human breast cancers, myoepithelial proliferation is a main feature of some of the common CMGT sub-types including complex carcinoma and malignant myoepithelioma.[51] Finally, dogs frequently develop mixed carcinomas which include a benign mesenchymal component usually present in the form of bone, cartilage or adipose tissue.[51] The Elston and Ellis method does not provide specific guidelines to grade carcinomas with multiple epithelial proliferation patterns, significant myoepithelial proliferation or neoplastic mesenchymal components.

Table 1.2 Scoring and grading system for simple carcinomas.

Source: Elston and Ellis. (1991)

	Tubule formation	Nuclear pleomorphism	Mitoses per 10 HPFs
1 point	> 75%	Absent	< 9
2 points	10 -75%	Moderate	9 - 17
3 points	< 10%	Marked	> 17
Sums of scores	3 -5	6 -7	8 -9
Grade	I	II	III

The recent CMGT grading method proposed by Pena et al. (2013), which is again a modified method of the Elston and Ellis method, addresses some of these shortcomings and better caters for grading complex and mixed carcinomas.[104] According to the Pena method, in heterogeneous carcinomas, tubular scoring is assessed in the most representative malignant area. In complex and mixed tumours, the percentage of tubule formation is scored considering only epithelial areas, and nuclear pleomorphism is evaluated in all the malignant components.

1.6 Treatments for CMGTs

Surgical excision is the most widely used method of treatment for CMGTs.[140,137] The extent of the surgical procedure varies from simple lumpectomy to staged bilateral mastectomies depending on the tumour characteristics and goals of treatment. For example, the goal of surgery for small, solitary neoplasms with less invasive behaviour is to excise them with clean margins by simple lumpectomy. In contrast, veterinary surgeons practice bilateral mastectomy when dogs have multiple neoplasms or neoplasms that are large or invasive to excise the current tumour or tumours with clean margins while preventing development of new tumours in future.[137,140] Surgical excision is curative for all benign tumours and some malignant CMGTs. However, 40%–50% of dogs that undergo surgical excision of malignant tumours experience post- surgical tumour recurrence and metastasis.[138] Post-surgical adjuvant therapy may be useful in these patients to prevent tumour recurrence and metastasis. The adjuvant therapies used for CMGTs include chemotherapy, cyclooxygenase (COX) inhibitors, desmopressin, anti-hormonal agents and immunotherapy with monoclonal antibodies.[66] Chemotherapy is the most used adjuvant therapy and commonly used chemotherapeutic agents include 5-Fluorouracil, Cyclophosphamide, Gemcitabine and Doxorubicin.[66,8] However, chemotherapy, has not shown to be particularly effective in reducing the rate of tumour metastasis in malignant CMGTs. Other adjuvant treatment modalities have been rarely used and therefore the efficacy of them is uncertain.[66] Moreover, without accurate prognostic markers, it is difficult to determine which excised

neoplasms should receive adjuvant therapy and which are unlikely to metastasise and therefore are unlikely to benefit from additional treatments.[140]

1.7 Prognostic factors of CMGTs

Prognostic factors are clinical, pathological or molecular measures that are used to predict the behaviour of neoplasms and the subsequent disease outcome.[47] Prognostic factors range from simple measures such as the diameter of a tumour to more complex indicators like molecular markers of cell division.[47] Disease-free interval (DFI) and overall survival (OS) are two commonly used determinants of disease outcome.[47] Disease-free interval is the length of time between the primary treatment and the first sign of recurrence.[47] Overall survival is the time from the first diagnosis of neoplasia to death from any cause, or to the end of the follow-up period.[47] Univariate analysis and multivariate analysis are two statistical analytical methods commonly used to determine the effect of prognostic factors on the disease outcome. A univariate analysis determines the effect of a single prognostic factor towards disease outcome.[79] In contrast, a multivariate analysis considers the effect of multiple prognostic factors at the same time.[79] Therefore multivariate analysis facilitates the recognition of influence of other prognostic factors, towards the prognostic factor of interest.[79] If the disease outcome determined by a prognostic factor is not significantly affected by other prognostic factors, it is considered an independent prognostic factor.[79] Consequently, an independent prognostic factor can determine the disease outcome reliably, even in the absence of information regarding other prognostic factors.[79] This makes independent prognostic factors particularly useful in clinical medicine.[79] A range of clinical, pathological and molecular prognostic factors with varying prognostic capacity have been described for CMGTs. However only a few have been identified to be independent prognostic factors.

1.7.1 Clinical prognostic factors

1.7.1.1. Age

Whether it is possible to use the age of a dog to determine the disease outcome of CMGTs is currently uncertain. Advanced age of the dog was reported to be associated with a shorter DFI and OS in five studies.[57,106,2,16,95] Of these, only two studies identified age as an independent prognostic factor by multivariate analysis.[2,95] However, several other studies have reported that age does not influence DFI or OS. [108,155,25,31] One reason for this observed variability may be that the age composition of the canine populations used, varied between studies. In addition, most of the studies have not clearly documented whether they used the age at diagnosis of mammary neoplasia or the age when the dog owner has first noticed the mammary tumours. This could be another reason for the apparent discrepancy observed between results.

1.7.1.2. Lymph node metastasis

Initial metastasis of CMGTs occurs through the lymphatic system. However, contradictory findings have been reported on lymph node metastasis and disease outcome of CMGTs. Four studies have reported that, lymph node metastasis was associate with poor disease outcome in dogs, using univariate analysis.[155,95,25,2] However, two studies which have used multivariate analysis, failed to demonstrate any relationship between lymph node metastasis and DFI or OS in dogs with CMGTs.[31,88,126] All these studies used cytology to determine lymphatic metastasis. However, cytology is not considered to be a highly sensitive method to detect tumour cells in lymph nodes as cytological examination only include a small area of a lymph node which may not include metastatic cells.[128] This might be one possible reason for the observed difference in results between studies. When cancer metastasis occurs via the lymphatic system, the lymph node or group of lymph nodes that the cancer cells first reaches are “sentinel” lymph nodes.[76] In human breast cancers, sentinel lymph nodes are well recognized.[76] Dogs have five pairs of mammary glands and the lymphatic drainage is more complex than in humans. Therefore, the knowledge about sentinel lymph nodes is limited in dogs and currently there is no consensus among veterinary clinicians about which lymph nodes should be sampled to

detect tumour metastasis. Most studies which have evaluated the prognostic significance of lymph node metastasis have not documented which lymph nodes were evaluated suggesting another possible reason for the observed difference in results between studies. In addition, micrometastases in lymph nodes are difficult to recognise using light microscopy and immunohistochemistry (IHC) or more sophisticated methods including positron emission tomography (PET) scanning which are not readily available for dogs may be required to identify these. Therefore, results between studies may also vary according to the methods used to identify tumour cells present in lymph nodes.

1.7.1.3 Tumour staging

A staging system for canine mammary tumours was first developed by Owen and colleagues in 1980 based on the staging system used for human breast cancer.[100] This staging system considers three components: tumour size (T), lymph node status (N), and distant metastasis (M) when determining the clinical stage of a mammary neoplasm (Table 1.3).[100] A simplified version of this system was later proposed by Rutteman et al. in 2001.[140] Many studies have shown that both systems provide consistent prognostic information regarding DFI and OS in dogs with mammary tumours. [159,57,25,3] In addition to providing prognostic information, tumour staging is an important determinant when making treatment decisions.

Table 1.3 Canine mammary gland tumour staging

Source: Owen et al. (1980)

Stage	Size	Regional metastasis	Distant metastasis
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
Stage IV	Any size	N1	M0
Stage V	Any size	N0 or N1	M1

Key: T1: ≤ tumour diameter 3 cm; T2: tumour diameter: 3—5 cm; T3: tumour diameter ≥ 5 cm; N0: without lymph node metastasis; N1: metastasis in lymph node present; M0: absence of distant metastases; M1: presence of distant metastases.

Canine mammary gland tumours include a range of neoplasms of various histological sub-types and grades with highly variable biological behaviour.[139] Some recent studies suggest that, although the current CMGT staging system provides a useful anatomical assessment of the extent of tumour spread within the body, it is not able to capture the biological and histological diversity within each stage which may lead to inaccurate prognostication for individual patients.[139,26] For example, it is possible for two tumours in the same stage not have the same disease outcome if one tumour is a low-grade tumour while the other is a high-grade tumour. Similarly, two tumours of the same stage may not have the same prognosis if one tumour belongs to a histological sub-type such as mixed mammary carcinoma which is less likely to metastasise while the other is a simple carcinoma which is more likely to metastasise.[139] In addition, other biological factors, including hormone receptor expression or cellular proliferation indices may also vary within each stage and influence prognosis.[139] Therefore, the currently available clinical staging systems need updating to include prognostic factors which may capture more of the biological behaviour of the tumours which may help to more accurately predict the disease outcome of CMGTs.

1.7.2 Reproductive status related prognostic factors

The prognostic significance of several reproductive status variables has been analysed in many studies. Whether these factors can be used as reliable prognostic indicators is uncertain due to contrasting results observed in different studies. Dogs with short oestrous cycles and a low number of oestrous cycles were reported to have shorter OS and DFI for CMGTs by univariate analysis.[18] However, the prognostic significance of these factors was lost on multivariate analysis in another study.[104] Ovariohysterectomy during mammary tumour excision was shown to be predictive of longer OS and DFI in univariate analysis by some authors.[155] while the same variable was shown not to affect either OS or DFI in several other studies. [130,57,37] Other reproductive variables including, number of pregnancies, age at first full term pregnancy and pseudopregnancy, have been analysed for their prognostic significance by many authors. However, none of these factors were shown to affect the post-surgical DFI or OS in dogs with mammary gland tumours. [31,37,57,129]

1.7.3 Tumour related prognostic factors

Tumour related factors including the tumour size, number of malignant tumours in a single animal at the time of presentation, surface ulceration, fixation to the underlying tissues and tumour growth rate have been evaluated in many prognostic analysis studies.[57,25] Of these, tumour size is the most widely evaluated tumour- related prognostic factor.[42,57] According to the findings of several studies, the larger the tumour size the shorter the DFI and OS. However, many of these studies evaluated the prognostic significance by univariate analysis only. [42,48,88,155,3] When the prognostic significance was evaluated by multivariate analysis in three studies, tumour size was found to be prognostic only in one.[57,25,108] Therefore, the ability of tumour size to reliably predict the outcome of CMGTs is still uncertain. This uncertainty is further complicated by the marked differences observed in the methods used by various authors to classify the tumours into “large”, “medium” and “small” categories. The most widely used method of classification is the separation of tumours as T1 (0—3 cm), T2 (3—5 cm) and T3 (> 5 cm) according to the WHO guide lines. However, a study by Philibert et al. (2003) used only two size-categories:

> 3 cm diameter tumours and < 3 cm diameter while Misdrope et al. (1976) used 4 size-categories: > 15 cm, > 10—15 cm, > 5—10 cm, > 0—5 cm. [108,88]

Rapid and invasive growth, ulceration of the skin, fixation to underlying tissues, location of the tumour, and the presence of additional mammary tumours have also been assessed for the prognostic significance by univariate and multivariate analyses. [42,57,126,159,3] Of these, ulceration of the skin overlying the tumour and rapid and invasive growth rate have been associated with shorter DFI and OS in dogs with mammary gland tumours.[42,126] Moreover, the study by Pena et al. (1998) found a significant association between tumour ulceration and metastasis.[106] However, the prognostic significance of surface ulceration was identified only by univariate analysis.

1.7.4 Histological prognostic factors

1.7.4.1 Histological sub-type

The prognostic value of histological tumour sub-types in CMGTs is inconclusive for several reasons. Firstly, there are studies which describe contrasting disease outcomes for the same histological sub-type. [115,116,65] For example, in one study, OS of dogs with simple tubular carcinoma was reported to be considerably shorter that the OS of dogs with complex carcinoma. However, in another study, the same group of researchers reported that OS of dogs with simple tubular carcinoma was not significantly different from OS of dogs with complex carcinoma.[115,116] The authors of this study attributed this discrepancy to the presence of a higher proportion of grade I simple tubular carcinomas in the latter study compared to the first. Grade I carcinomas usually have a better prognosis compared to grade II or III carcinomas. However, the results of these two studies indicate that histological tumour sub-type may not provide reliable prognostic information when it is used as the only prognostic indicator.

Secondly, although there are many histological sub-types of CMGTs described under different classifications, the prognostic significance is only well reported for a few sub-types including simple carcinoma and mixed mammary tumours. For other sub-types,

either limited prognostic information is available or the prognostic significance is unknown. This is mostly due to the low prevalence of some histological sub-types such as carcinosarcoma, myoepithelial sarcoma, mucinous carcinoma, lipid rich carcinoma compared to others making meaningful studies and data collection difficult.[115,22] Consequently, many prognostic studies contain few or none of the less common CMGT sub-types. In fact, one of the largest prognostic studies conducted by Hellmen et al. (1993)[57], included only one uncommon sub-type, namely malignant myoepithelioma. The prognostic significance of malignant myoepithelioma was not analysed in this study due to the low number of cases recruited. Similarly, only six uncommon sub-types were included in the largest of the four studies evaluating the prognostic significance of tumour sub-types described in the 2011 Goldschmidt classification. In that study, the prognostic significance of two of the uncommon sub-types, including, lipid-rich carcinoma and micropapillary invasive carcinoma, were not evaluated due to inadequate numbers of cases.[115,116,62,104] Some researchers have attempted to form broad categories of mammary tumours, by collating several sub-types sharing common features together. In one such study conducted by Misdrope et al. (1976)[88], all CMGTs included were considered in three broad categories: simple carcinoma, complex carcinoma and sarcoma. In this study, dogs in the sarcoma category had the shortest OS while dogs with complex carcinomas had the longest OS. However, prognostic information revealed by this study was general and therefore has a limited prognostic value.

In addition to the prior mentioned ambiguities of the prognostic significance of histological classification of CMGTs, currently there is no consensus on which histological sub-type classification system best predicts the disease outcome of CMGTs.[23] So far, only a single study has compared the prognostic ability of different classification systems.[20] Further, rare types of CMGTs, such as mammary carcinoma with sebaceous differentiation, lobular carcinomas, and pleomorphic lobular carcinomas which were not discussed in the Misdorpe classification or Goldschmidt classification are published in veterinary literature.[23] Prognostic determination of these sub-types is challenging, and it is highly unlikely to achieve satisfactory prognostic guidelines for these rare tumour entities without a multi-institutional approach.[115]

1.7.4.2 Histological grade

Although tumour grade is considered as one of the best prognostic indicators for human breast cancers, the utility of tumour grade as a prognostic factor for canine MGTs is not well established. [104] Two studies have evaluated the prognostic significance of the Elston and Ellis method when used to grade CMGTs. In the first study conducted by Misdrop et al. (1976), the grade I category had a significantly lower percentage of tumour related deaths compared to grade III category.[88] However, percentage tumour related deaths in the grade II category was not significantly different either from grade I or grade III categories. In this study, most of the tumours categorised as grade I or grade III tumours were simple carcinomas while the neoplasms categorised as grade II included the least number of simple carcinomas. The authors of this study attributed this weak distinction between grade II category and other categories in terms of percentage tumour related deaths to the lower proportion of simple carcinomas included in the grade II category. Further, based on this assumption, it was suggested that grading of canine mammary neoplasms using Elston and Ellis method has a prognostic utility when it is used to grade simple carcinomas while it is least useful with other histological sub-types. The other study by Karayannopoulou et al. (2005) mostly included simple carcinomas in all grade I, grade II and grade III categories.[65] In this study, survival intervals were significantly shorter in dogs diagnosed with grade III tumours than the dogs diagnosed with either grade I or grade II tumours. The dogs with mammary gland tumours categorised as grade I had the longest average survival time. However, in the grade II category, nearly 50% of the dogs were dead at the end of the 2-year follow-up period suggesting that dogs with grade II tumours were equally likely to live as to die making this grade poorly prognostic of disease outcome for malignant CMGTs.[65]

More recently Peña et al. (2013) modified the original Elston and Ellis method for grading CMGTs.[104] This canine-adopted Pena method caters for grading complex and mixed carcinoma in addition to simple carcinomas. However, several studies have revealed that, even using this updated method, there is no significant difference in the disease outcome for dogs with grade II mammary neoplasms when compared to grade I neoplasms.[115,80] In these studies, both grade I and grade II tumours behaved similarly with prolonged overall survival, infrequent local recurrence and distant metastasis. Therefore, in this modified grading system, the prognostic utility of the grade II category is questionable.

Many studies reported satisfactorily low inter- and intra-observer variability of Elston and Ellis method when it is used to grade human breast cancers. Therefore, currently tumour grade determined by Elston and Ellis method is recommended to use in prognostic determination for human breast cancers.[114] However, no studies have determined the inter- and intra-observer variability of Elston and Ellis grading method when it is used to grade CMGTs. Two studies have reported a considerably higher degree of inter-observer variability in the grading method proposed by Peña et al (2013).[104] Except for these two studies, no other studies have evaluated the CMGT grading methods regarding inter and intra-observer variability. Therefore, considering the shortcomings and insufficient validation information on currently available grading methods, the definite utility of tumour grade as a prognostic factor for CMGTs is currently uncertain.

1.7.5 Molecular prognostic markers

A molecular marker for cancer is a molecular entity (DNA, RNA, or protein) which can be isolated from biological materials to obtain a quantitative measurement of biological homeostasis in cancer-affected cells, tissues, or an organism.[121] In human breast cancers, the prognostic potential of molecular markers has been extensively studied.[124] Many molecular markers, particularly, steroid hormone receptors, cell proliferation markers, tumour suppressor genes and oncogenes and enzymes such as cycloxgenase-2 (COX-2) have been identified as reliable prognostic factors for breast cancers. However, in veterinary medicine, only a few studies have been conducted to evaluate the prognostic potential of these molecular markers and they are not currently routinely used to determine prognosis for CMGTs.[106,161,31]

1.7.5.1 Steroid hormone receptors: progesterone receptor (PR) and oestrogen-receptor-alpha (ERα)

In the last decade, the utility of steroid hormone receptors as prognostic factors for human breast cancers has been investigated in-depth. Progesterone receptor (PR) and oestrogen-receptor-alpha (ERα) are the most widely explored steroid hormone receptors in breast

cancers. Patients with ERα-positive breast cancers have been shown to have a better response to hormonal therapy and therefore these neoplasms have a better prognosis than patients with ERα-negative breast cancers.[109] Expression of PR was detected more frequently in well-differentiated breast carcinomas than in poorly differentiated ones and tumours with higher number of PR receptors were reported to respond better to tamoxifen therapy.[10] Studies evaluating the prognostic significance of ERα and PR expression in canine MGTs are limited and include one retrospective study[24] and two prospective studies.[95,31] The findings of these studies are inconclusive. In one prospective study, increased immunostaining for ER or PR receptors in malignant CMGTs was associated with a better prognosis. However, a similar association was not observed in the other prospective study. The increased presence of PR was associated with an increased OS in the retrospective study.[24] The value of ER and PR immunostaining as a predictive marker of favourable response to hormone therapy has been evaluated in one study of CMGTs. In this study, the presence of ER or PR receptors on the neoplastic cells did not have any impact on the response of neoplasm to hormone therapy.[53]

1.7.5.2 Markers of cell proliferation: Ki-67 and proliferating cell nuclear antigen (PCNA)

Uncontrolled cellular proliferation is a key feature of cancer development and progression. The nuclear proliferation marker Ki-67 is one of the most researched molecular biomarkers for prognostic determination in human breast cancers and many studies have suggested that Ki-67 immunostaining can be effectively used to predict prognosis in breast cancers.[30,63,97] Despite the studies showing prognostic significance of Ki-67 in human cancers, the American Society of Clinical Oncology does not recommend its use in clinical practice due to the lack of uniformity in laboratory techniques and analysis.[110] Few canine studies have investigated the presence of Ki-67 in mammary gland tumours. In these studies, presence of high Ki-67 immunostaining was positively correlated with the development of metastasis as well as shorter DFI and OS.[106,126] However, these studies have included relatively low numbers of samples. In addition, levels of Ki-67 in older dogs were shown to be lower than in younger dogs, probably because cell proliferation in general is slower at older age.[106] Further, in contrast to human breast cancer studies, no studies have evaluated the inter-observer variability and inter-laboratory variability in Ki-67

immunostaining in CMGTs and cut-offs relevant to clinical decision making have not been determined for CMGTs. Therefore, the utility of Ki-67 for prognostic determination in CMGTs is currently uncertain and more studies with a large number of samples are necessary for confirmation.

Proliferating cell nuclear antigen (PCNA) is another marker of cell proliferation used as a prognostic factor in human breast cancers. However, in contrast to human breast cancers, PCNA immunostaining was not associated with tumour metastasis, recurrence or DFI in dogs with mammary neoplasms.[106]

1.7.5.3 Tumour suppressor genes: p53

The gene p53 codes for a protein that regulates the cell cycle and therefore functions as a tumour suppressor gene. Mutations in p53 gene lead to uncontrolled cellular proliferation ultimately causing cancer.[164] Regarding the prognostic significance of p53, mutations in the p53 gene have been shown to be associated with a significantly shorter DFI and OS in human breast cancer patients.[164] However, p53 expression has been shown to vary between different sub-types of human breast cancers.[102] Additionally, some studies have demonstrated that increased p53 expression correlates with longer DFI and OS of breast cancer patients. Therefore, currently the prognostic utility of p53 in human breast cancers is unclear. A limited number of studies have investigated the prognostic significance of p53 expression in canine mammary neoplasms with contrasting results.[36,94] Therefore, similar to human breast cancers, the prognostic utility of p53 in canine mammary neoplasms is currently uncertain.[94,73]

1.7.5.4 Oncogenes: human epidermal growth factor receptor-2 HER-2

An oncogene is defined as a gene that encodes a protein that is capable of transforming cells in culture or has the potential to cause cancer.[28] Human epidermal growth factor receptor-2 (HER-2) is a cell surface protein encoded by HER-2 gene oncogene. It is used as a prognostic marker in human breast cancers. Patients with HER-2-positive breast cancers are shown to have a poorer prognosis than patients with HER-2-negative breast cancers.[144]

The prognostic function of HER-2 in CMGTs is uncertain as some studies have demonstrated that HER-2-positive malignant CMGTs have a worse prognosis, while other studies found that dogs with HER-2-positive neoplasms actually had a better prognosis than dogs with HER-2 negative tumours.[105]

1.7.5.5 Cyclooxygenase-2 (COX-2)

Cyclooxygenase-2 or COX-2 is an enzyme that in humans is encoded by the Prostaglandin-endoperoxide synthase 2 (PTGS2) gene. Recent studies have investigated the presence of cyclooxygenase-2 (COX-2), an enzyme involved in inflammation, as a prognostic marker in human breast cancers as well as in CMGTs. In human breast cancers, increased immunostaining for COX-2 was associated with a poor prognosis.[118] A limited number of studies have similarly investigated the prognostic significance of COX-2 in CMGTs. In one study, increased COX-2 immunostaining was significantly associated with reduced tumour differentiation, suggesting that neoplasms with increased COX-2 may have a poorer prognosis.[86,71] This was supported by another study that observed increased immunostaining of COX-2 was associated with lymph node metastasis at the time of surgery, development of distant metastasis during the follow-up, and shorter DFI and OS.[111,71] However, as these observations were only tested by univariate analysis, it is currently unknown whether that COX-2 can be used as an independent prognostic factor in CMGTs. In summary, the prognostic significance of many molecular markers tested so far for CMGTs is either inconclusive or show conflicting results.

1.8 Canine mammary gland tumours and human breast cancers

In recent decades, many studies have compared mammary neoplasia of humans and dogs and identified many clinical and molecular similarities between them.[112] The clinical similarities include spontaneous occurrence of tumours, hormonal aetiology, and age of onset. Both women and intact female dogs develop spontaneous mammary neoplasms that are thought to be influenced by reproductive hormones.[112,14] When adjusted for the life spans of the two species, the average age at onset of mammary neoplasia in humans

(after 40 years) is approximately the same as dogs (after 6 years) with the peak incidence of the disease is also comparable between the two species.[126] The clinical course of mammary tumours also similar but not identical in dogs and humans. In dogs, approximately 50% of the mammary neoplasms are malignant while 20-50% of malignant neoplasms metastasising to regional lymph nodes and lungs. Bone metastasis is infrequent. Approximately 50%-70% of human breast lesions are benign neoplasms.[52,99] Nearly 20% of women with breast cancers develop metastasis to lymph nodes and lung with frequent bone metastasis.[122] In both species, a larger tumour size, presence of lymph node metastases and advanced clinical stage are linked with a worse prognosis.[112]

Despite these similarities, CMGTs are histologically more variable than human breast cancers. The most common histological type of mammary neoplasm in women is invasive ductal carcinoma while other histological sub-types are less frequent.[112] In contrast, CMGTs may originate from different types of tissues in the mammary gland including epithelial or mesenchymal tissues and the most common histological sub-types include simple carcinoma and mixed mammary tumours. Many recent studies have compared CMGTs and human breast cancers on a molecular level and these studies have identified similarities regarding steroid receptors, proliferation markers, epidermal growth factor, p53 suppressor gene mutations, metalloproteinases, and cyclooxygenases, among many others.[112] Based on these clinical and molecular similarities it has been suggested that canine mammary tumours are a valid model to study human breast cancer.

1.9 Tumour microenvironment (TME) and tumour associated inflammation (TAI)

Recent research in the field of cancer biology has suggested that while genetic alterations in tumour cells are essential for tumour development, the cancer stroma also plays a critical role in tumour progression and metastasis.[5] Cancer stroma is the tissue surrounding the tumour cells in a tumour which is composed of many cellular and non-cellular components collectively known as the tumour microenvironment (TME).[5] In addition to the non-cellular matrix composed of fibrous proteins, glycoproteins, proteoglycans, and

polysaccharides, the TME includes many cellular components including immune-inflammatory cells, fibroblasts, myofibroblasts, neuroendocrine cells, adipose cells, and the blood and lymphatic vascular networks.[5] Inflammation is the main process which maintains and regulates the crosstalk between the TME and the tumour cells. Within the TME, inflammation influences every aspect of tumour development and progression, as well as response to therapy.[123] In fact, inflammatory components within the TME, termed as tumour associated inflammation (TAI), can promote an anti-tumour immune response or support tumour progression and metastasis.[123] The key features of TAI include leukocyte infiltration in response to cytokines or chemokines, angiogenesis and tissue remodeling.[5,156] Given the importance of TAI, recent cancer studies have been aimed at identifying the key inflammatory mediators in the TME which can better predict the tumour behaviour. Additionally, a better understanding of TAI could also be important for cancer treatment as the TAI response can be modified by novel therapeutics, potentially changing the biological behaviour of cancers, to achieve a better disease outcome. Interestingly, some reports have concluded that prognostic markers related to TAI are in fact better predictors of patient survival than other conventional prognostic factors that are currently used for various human cancer types.[21,5] In human medicine, currently there are three key research areas which are focused on identifying TAI related prognostic markers and therapeutic targets. These include immune cell infiltration of tumours[152], chemokines and their receptor expression by tumour cells[165], and aberrant expression of immune checkpoint molecules by tumour cells.[156]

1.9.1 Prognostic significance of immune cell infiltration in cancers

The three components: quantity, functionality, and the localisation of immune cells within the TME are collectively identified as the "immune contexture" of a cancer.[45,11] Many human studies have extensively investigated the immune contexture of different types of cancers and revealed that it can have a significant impact on cancer recurrence, metastasis and patient survival. For example, in colorectal cancers the presence of a high number of memory T-cells in the invasive front and within the centre of the neoplasm correlated with later metastasis, longer OS and DFI of the patients.[45] In ovarian carcinoma, the presence of

an increased number of intra-epithelial T-lymphocytes was associated with longer OS times of the patients.[60] Increased infiltration of tumour stroma by CD8+ lymphocytes was associated with longer OS times in patients with non-small cell lung cancers.[50] In hepatocellular carcinoma, increased intra-tumoural and peri-tumoural infiltration of FOXP3+ regulatory T-cells was associated with shorter DFIs and OS times of the patients.[11,50] Some of these studies have compared the prognosis suggested by the immune cell infiltration with other conventional prognostic factors including tumour size, tumour grade and clinical stage of the tumour and found that immune cell infiltration often better predicts disease outcome than conventional prognostic indicators. In human breast cancer, the presence of various types of tumour infiltrating immune cells including macrophages[142], T-cells[162,132,70] and mast cells[113] have been correlated with the disease outcome of the patients.

Prognostic significance of mast cell number and distribution in cancers

Mast cells are an important innate immune cell type frequently found in the inflammatory cell infiltrates of many cancers.[83,32] Mast cell-derived mediators can either be pro-tumourigenic, causing tumour progression and metastasis, or anti-tumourigenic limiting tumour growth.[86] The tumour promoting functions of mast cells are attributed to different types of angiogenic factors, proteases and growth factors secreted by them.[32,83,98] These pro-tumourigenic mediators secreted by mast cells promote tumour progression, either by direct effect on tumour cells, or indirect influence on TME. Regarding the indirect effect of mast cells on TME, the pro-tumourigenic mediators secreted by mast cells can alter the TME in favour of tumour growth and metastasis by enhancing tumour angiogenesis and remodelling of tumour matrix.[98] The anti-tumour effects of mast cells are mostly attributed to the TNF-α, IL-4 and IL-9 that they secrete. These cytokines stimulate the immune system enhancing tumour cell destruction, limiting the growth of neoplasms.

The degree of mast cell infiltration is shown to be predictive of disease outcome in some human cancers.[83,152] The majority of reports have correlated increased mast cell infiltration with a unfavourable prognosis, [82,91,145] although a few studies have associated mast cell infiltration with a better disease outcome.[15,43] However, most of these studies have

measured the overall mast cell density irrespective of their location. Some recent studies have suggested that presence of mast cells at different locations within a tumour may have distinct roles in tumour progression in cancers. Further, these studies have proposed that rather than the overall mast cell density, mast cell density in different tumour locations to be better prognostic indicators for some human cancers.[7] One such large study on invasive breast cancers showed that patients with higher stromal mast cell density had better disease outcomes including longer OS and DFI than patients with lower stromal mast cell density.[113] In addition to the prognostic utility of mast cells in breast cancers the potential of using them as therapeutic targets has also been considered. Experimental studies carried out with murine models suggested that mast cells could be used as potential therapeutic targets for breast cancers. These studies indicated that compounds which promote controlled release of anti-tumourigenic mediators from mast cells may enhance anti-tumour immunity.[98]

Limited numbers of studies have explored mast cells in CMGTs and the role of mast cells in prognosis of CMGTs is currently not well known. One study reported a positive correlation between mast cell density and micro-vessel density in malignant mammary tumours suggesting a contribution of mast cells towards tumour angiogenesis.[72] Im et al. (2011)[61] demonstrated similar findings and further suggested that increased amount of the enzyme tryptase produced by mast cells may have promoted tumour angiogenesis.[61] Although these studies provide some useful clues about the role of mast cells in CMGTs, none of these studies investigated the prognostic significance of mast cell distribution in CMGTs using retrospective or perspective survival analysis studies. Therefore, it may be useful to investigate the prognostic potential of tumour infiltrating mast cells in CMGTs.

1.9.2 Prognostic significance of chemokines and chemokine receptors in cancer

Chemokines are low molecular weight signalling proteins that are produced by innate immune cells such as eosinophils, neutrophils, macrophages, dendritic cells, and natural killer cells. Chemokines function to regulate the trafficking and positioning of the cells of the immune system by activating trans-membrane G protein-coupled chemokine receptors

located on the surfaces of many inflammatory cells and some tissue cells.[96] All chemokines contain the amino acid cysteine in them. According to the spacing pattern of the first two cysteines in their sequence, there are four structural sub-types of chemokine; C, CC, CXC and CX3C.[165] Functionally, chemokines are categorised either as homeostatic or inflammatory.[165,92] Homeostatic chemokines are constitutively produced by certain inflammatory and tissue cell types and assist the host immune system in recognising transformed cells. In contrast, inflammatory chemokines are induced by inflammatory stimuli to attract neutrophils and eosinophils from the circulation to the site of infection or injury.[92,165]

To date, nearly 50 chemokines have been identified. Twenty chemokine receptors, which are expressed mainly by leukocytes, have been identified along with four atypical chemokine receptors which are expressed by non-leukocyte cell types, including erythrocytes, and vascular endothelial cells.[101] Of the 24 chemokine receptors, six are known to bind to a single chemokine while other receptors have affinity to multiple chemokines.[165] Some tumour cells were also shown to aberrantly express chemokines and chemokine receptors on their cell surfaces. More than half of the chemokine receptors have been known to be important in tumour biology, especially tumour growth and metastasis.[165] The recognition of the role of chemokines in tumour biology suggested that chemokine receptors could be potential therapeutic targets for the treatments of some cancers.[96,92]

In human breast cancer, intra-tumoural chemokine expression has been found to influence tumour growth, angiogenesis and distant metastasis.[50] Studies of human breast cancer have suggested that expression of chemokines and chemokine receptors may be useful to predict the behaviour of neoplasms, especially the development of distant metastasis.[4,92] Chemokine receptors, including CXCR2, CXCR3, CXCR4, CXCR7, CCR4 and CCR9 were shown to influence cancer cell survival and proliferation, tumour angiogenesis, and development of resistance to conventional and targeted therapies in human breast cancers.[96,165] Further, targeting chemokines and chemokine receptors using monoclonal antibodies developed against them has been reported to show promise as therapy.[4] In dogs, little research has been done investigating chemokine expression in CMGTs. The following table summarises

the details of four chemokine receptors and four chemokines identified as useful prognostic indicators in human breast cancers and the research findings investigating the same receptor and ligands in CMGTs. In summary, the prognostic significance of the chemokines studied in human breast cancers show variable prognostic significance in CMGTs.

Table 1.4 Chemokines and chemokine receptors associated with disease outcome of human breast cancers and their expression in canine mammary gland tumours.

Chemokine receptor/ligand	Known physiological function	Prognostic significance in human breast cancer	Expression/prognostic significance in canine mammary gland tumours
CXCR4	Regulate lymphopoiesis, myelopoiesis, and T-cell migration	Increased gene expression or immunostaining correlates with increased metastatic potential.[4,150]	Increased gene expression in malignant neoplasms compared to normal mammary gland.[68]
CXCR7	Scavenging of CXCR4 and CXCL11 receptors	Increased gene expression of CXCR7 correlates with a poor prognosis and co-expression of CXCR4 and CXCR7 genes correlates with reduced metastasis. [4,85]	Increased gene expression in malignant neoplasms compared to normal mammary gland.[68]
CXCL12	Regulate haematopoiesis, T-lymphocyte migration and angiogenesis	Increased gene expression or immunostaining correlates with metastasis.[87,46]	Lower gene expression in tumour stromal cells of mammary carcinoma than stroma of adjacent non-neoplastic mammary gland tissues.[39]
CXCR3	Regulate T-lymphocyte trafficking and function	Increased gene expression or immunostaining correlates with distant metastasis.[75,163]	Increased gene expression in metastatic mammary neoplasms than non-metastatic neoplasms, benign neoplasms, and non-neoplastic mammary gland.[19]
CCR4	Regulate T-lymphocyte and dendritic cell migration and lymphopoiesis	Increased gene expression or immunostaining correlates with increased LN metastasis.[74]	Not studied in canine MGTs.
CCR9	Regulate T-lymphocyte homing to gut	Increased immunostaining correlates with increased tumour invasion and metastasis. [4,64]	Not studied in canine MGTs.
CXCL10	Regulate T-lymphocyte migration and involve with adaptive immune responses	Increased gene expression correlates with poor prognosis.[92,93]	Increased gene expression in malignant tumours compared to adjacent non-neoplastic mammary gland.[68,69]
CCL5	Regulate T-lymphocyte and monocyte migration and innate and adaptive immune responses	Increased gene expression correlates with distant metastasis.[4,153,158]	Significantly higher gene expression in simple adenoma and tubulopapillary carcinoma than adjacent non-neoplastic mammary gland.[6]

1.9.3 Prognostic significance of immune checkpoints and chemokine receptors in cancer

The host immune system continuously monitors the body to detect and destroy infected or neoplastic cells via a process known as immunological surveillance.[156] However, immunosurveillance may lead to uncontrolled immune responses which may cause severe damage to the host tissues.[162] To prevent this, the magnitude of the host immune response is regulated by a balance between co-stimulatory and co-inhibitory signals.[162] The co-stimulatory signals promote the host immune response while co-inhibitory signals suppress the host immune response. These signals, which are generated through receptor-ligand interactions, are collectively referred to as immune checkpoints.[156] The immune checkpoint receptors are usually located on the surfaces of immune and inflammatory cells and the ligands are produced by either inflammatory and immune cells or certain types of tissue cells. The co-inhibitory immune checkpoints are important in tumour biology, as tumour cells exploit these to evade the host immune response. Programmed death ligand 1 (PD-L1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) are two such co-inhibitory immune checkpoint molecule that are frequently expressed by cancer cells of humans and dogs.[156]

1.9.3.1 Programme death ligand-1 (PD-L1)

Usually, PD-L1 is present on the surface of macrophages, and the corresponding receptor, PD-1, is present on activated CD8+ T-cells.[156] These cells are the main mediators of immune-mediated cytotoxic cell lysis. The interactions between PD-L1 and PD-1 prevent indiscriminate destruction of normal tissue cells by CD8+ T-cells during immune mediated cytotoxic cell lysis. Inflammatory cytokines present in TME induce aberrant PD-L1 expression on tumour cells. The co-inhibitory signal generated by the interaction between PD-1 expressed on activated CD8 + T-cells and PD-L1 produced by tumour cells, suppresses the host anti-tumour immune response by inhibiting CD8 + cytotoxic T-cell mediated tumour cell destruction.[156]

In many studies of human cancers, PD-L1 was shown to be more frequently present on cells from malignant neoplasms than cells from benign tumours.[156,162] Based on this finding, the prognostic utility of PD-L1 in human cancers has been extensively investigated during the past few years. A recent meta-analysis of previously published studies showed a strong association between increased immunostaining for PD-L1 protein in cancer cells and shorter DFI and OS.[156] Another meta-analysis of five previously published studies on the presence of PD-L1 in breast cancers suggested that PD-L1 immunostaining is a promising biomarker for predicting the biological behaviour of human breast cancers.[162]

In addition to its role as a potential prognostic marker in human cancers, some studies have suggested that PD-1 and PD-L1 can be manipulated to achieve therapeutic benefits.[58] Monoclonal antibodies developed against PD-1 or PD-L1 proteins can block the receptor-ligand interaction *in vitro* or *in vivo*, restoring the host immune response against cancer cells in humans and dogs.[17] Currently, commercial preparations of anti-PD-1 and anti-PD-L1 monoclonal antibodies are available for therapeutic usage.[17] They are currently used as adjuvant immunotherapies in human patients with various types of cancers including breast carcinomas.[17]

Immune checkpoints are infrequently studied in dogs and there are currently only a few preliminary studies of PD-L1 gene expression and immunostaining in canine cancers. Two studies have identified the presence of PD-L1 in several canine cancers using immunohistochemistry.[77,133] According to these studies, PD-L1 is frequently detectible in melanoma, mammary gland tumours, mast cell tumour, prostate cancers and lymphoma, but less frequently present in different types of sarcomas including osteosarcoma, fibrosarcoma and haemangiosarcoma.[77,133] Of the two studies, all tumours included in one study were malignant tumours[133] while the other study included both malignant and benign tumuors.[77] The number of cases included from each tumour type as well as from benign and malignant categories were low in both studies. Therefore, differences in PD-L1 immunostaining among various tumour types and between benign and malignant tumours were not analysed. Moreover, none of these studies have evaluated the prognostic utility of PD-L1 using retrospective or prospective patient survival studies.

One recent study has investigated the therapeutic potential of PD-L1 blockade in dogs using monoclonal antibodies developed against canine PD-L1 protein.[78] This study included an *in vitro* assay and a pilot clinical study. The *in vitro* assay investigated the immunomodulatory effects of recombinant anti-PD-L1 monoclonal antibody using a canine tumour cell line.[78] In this assay, PD-L1 blockade was shown to significantly enhance cytokine production by tumour cells. In the pilot clinical study, seven dogs with oral melanomas and two dogs with undifferentiated sarcomas were treated with recombinant anti-PD-L1 monoclonal antibody. At the end of the treatment period subsequent tumour regression was observed in single cases from each tumour type.[78]

Given the prognostic and therapeutic efficacy of PD-L1 immune checkpoint in human breast cancer, it may equally be useful as a prognostic marker and therapeutic target in canine mammary neoplasms. However, the prognostic utility of PD-L1 or the therapeutic potential of PD-L1 blockade in canine MGTs have not yet been evaluated.

1.9.3.2 Cytotoxic T-lymphocyte antigen-4 (CTLA-4)

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a member of the immunoglobulin superfamily present on activated T-cells. Unlike PD-L1, CTLA-4 is usually present in intracellular vesicles and is only transiently present on the surface of activated T-cells. T-cells costimulatory protein-28 (CD28) is homologous to CTLA-4, and both molecules bind to CD80 and CD86 ligands present on antigen-presenting cells including macrophages and dendritic cells.[59] Cytotoxic T-lymphocyte antigen-4 binds with CD80 and CD86 with greater affinity than CD28 which enables it to outcompete CD28. Upon binding with the ligand, CTLA-4 transmits an inhibitory signal to T-cells. Additionally, CTLA-4 is also found in regulatory T- cells and contributes to their inhibitory function. There are two isoforms of human CTLA-4: a membrane-bound receptor isoform with both extracellular and intracellular domains and a secreted, soluble isoform, which only has the extracellular domain for ligand-binding.[59] These two isoforms reduce T-lymphocyte activation to maintain self-immune tolerance and homeostasis. Similar to human CTLA-4, canine CTLA-4 has also been shown to have a soluble isoform.[148]

In human medicine CTLA-4 has been shown to be expressed on some tumour cells in different types of cancers including melanoma, neuroblastoma, oesophageal carcinoma, lung cancer and human breast cancer. Despite the well-known immunosuppressive role of CTLA-4, the association between the presence of CTLA-4 and disease outcome is unclear, possibly due to the limited number of studies investigating this association.[160,131] While one study reported CTLA-4 immunostaining in human breast cancers to predict prognosis independent of other conventional prognostic factors including age, clinical stage, tumour histological grade, presence of tumour emboli, ER, PR or HER-2 status and Ki-67 immune staining[160], these findings were inconsistent with other similar studies. In veterinary medicine only a few studies have investigated CTLA-4 gene expression in canine neoplasms. One study comparing CTLA-4 gene expression in peripheral blood lymphocytes between dogs with histiocytic sarcoma, other neoplasms, and healthy dogs, reported that CTLA-4 expression was significantly higher in the peripheral blood lymphocytes of dogs with histiocytic sarcoma than in the other two groups.[147] Another study showed that CTLA-4 gene expression is prognostically important in canine high-grade B cell lymphomas.[146] No previous studies have investigated the presence of CTLA-4 either by gene expression or immunostaining or the prognostic significance of CTLA-4 in canine MGTs.

1.10 Summary

Mammary gland tumours are common in female dogs and a significant cause of disease and mortality, especially in countries where early spaying is not a routine practice. Risk factors for CMGT development include old age, prolonged exposure to reproductive hormones, breed, and obesity. Canine mammary gland tumours represent a group of highly heterogeneous neoplasms with variable biological behaviours. Approximately half of CMGTs are histologically classified as malignant and approximately 50% of the malignant neoplasms progress to develop distant metastasis. Many clinical, histological, and molecular prognostic markers are currently available to predict the behaviour of CMGTs. Of these prognostic markers, tumour size, tumour histological classification, and tumour grade are frequently used by veterinary pathologists and clinicians to predict the prognosis

of a CMGT. However, current prognostic determination methods based on histological features of the neoplasms including tumour histological classification and tumour grade are subject to marked inter and intra-observer variation. In addition, tumour staging which is considered as one of the best methods for prognostic determination has been shown not to accurately predict the biological behaviour of CMGTs. Further, some molecular methods have been suggested for prognostic determination in CMGTs, but none have been thoroughly investigated.

The main modality of treatment for CMGTs is surgical tumour excision and adjuvant therapies are infrequently included in CMGT treatment protocols. This is partly due to an inability to accurately identify which tumours would benefit from adjuvant therapy as well as the poor efficacy of currently available adjuvant therapies. Therefore, there is a need for more accurate prognostic markers to accurately determine the prognosis of CMGTs. Tumour associated inflammation in the tumour microenvironment influences the behaviour of many human cancers including human breast cancers. Some of the features of TAI were shown to predict the prognosis of human breast cancers more accurately than conventional prognostic markers. These features include tumour-infiltrating immune cells, chemokines and chemokine receptors, and immune checkpoint molecules present on tumour cells. Considering the similarities of clinical and molecular features of CMGTs and human breast cancers, these prognostic markers may similarly be prognostic for CMGTs.

The following chapters of this thesis will present research aimed at identifying inflammation-related prognostic markers for CMGTs. Chapter 2 and Chapter 3 will include the surveys carried out in Sri Lanka and New Zealand to identify the clinical and pathological features of mammary neoplasms in dogs in these two countries. Chapter 4, Chapter 5, and Chapter 6 will discuss the research work carried out to determine the prognostic significance of inflammation-related prognostic markers adopted from human breast cancers. The prognostic markers investigated in CMGTs include mast cell distribution - Chapter 4; chemokines and chemokine receptor expression by tumour cells - Chapter 5; and aberrant immune checkpoint expression by tumour cells - Chapter 6. Chapter 7 will provide a general discussion and directions for future research.

1.11 Bibliography

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Chapter 2 : Clinicopathological features of mammary gland tumours in dogs in Sri Lanka

2.1 Introduction

Prolonged exposure to female reproductive hormones promotes mammary carcinogenesis.[30] Consequently, mammary gland tumours are the most common neoplasm among intact dogs.[2,12] Ovariohysterectomy (OHE), which is the surgical removal of the ovaries in companion animals colloquially known as spaying performed at an early age minimises the prolonged exposure of mammary tissues to reproductive hormones, and thereby reduces the risk of mammary neoplasia.[17,32] As a result, the incidence of canine mammary gland tumours (CMGTs) is decreasing in the regions of the world where OHE is routinely performed at an early age.[29] Sri Lanka is a South Asian country where spaying of dogs at an early age is not a common practice.[6] Dog spaying in Sri Lanka is mostly conducted during mass de-sexing programs that are generally done on an opportunistic basis.[6] Therefore, most of the dogs in Sri Lanka are either intact or have been spayed at an older age, which predisposes them to mammary neoplasia. In fact, mammary gland tumours are one of the most common neoplasms of dogs in Sri Lanka and are an important cause of mortality in this population.

Given the importance of canine mammary neoplasia in Sri Lanka, it is necessary to improve the diagnostic, prognostic, and therapeutic aspects of CMGTs. In this thesis, tumour-associated Inflammation related prognostic markers were investigated to see whether these could be useful for veterinary pathologists and clinicians to better predict pronosis in dogs with mammary neoplasms. To achieve this, samples were collected as part of a one-year prospective survey conducted at two veterinary practices in Sri Lanka. Unfortunately, due to the inability to obtain follow-up data for some patients, CMGT cases obtained from Sri Lanka could not be used for prognostic analysis. However, the collected information was still valuable as no previous study has investigated mammary neoplasms in dogs in Sri Lanka. Therefore, the collected data was analysed with the aim of identifying

clinicopathological features of mammary neoplasms in female dogs in Sri Lanka. It was expected that these features could be a useful guide for Sri Lankan veterinary pathologists and clinicians to inform and possibly modify their current approaches to more accurately diagnose and effectively treat mammary neoplasms in dogs.

A knowledge of possible risk factors for CMGTs is important to help develop effective strategies to minimise the incidence of these neoplasms. Previous studies have suggested that dogs older than 7 years, small-sized dogs, obese dogs, and dogs that were spayed later in life but still nulliparous, are at increased risk of developing mammary gland tumours.[3,7,23,10] However, the relative impact of these risk factors appears to be variable within different regions of the world. Therefore, as a second objective, patient profile including age, breed, body condition score, reproductive status, and parity of the dogs included in the survey was analysed to identify possible risk factors for mammary neoplasia in dogs in Sri Lanka.

2.2 Material and methods

2.2.1 Sample collection

Samples for the present study were obtained from the Veterinary Teaching Hospital (VTH), Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, and a private veterinary practice in Colombo (VPC), Sri Lanka. All dogs that presented to these clinics between June 2016 and June 2017, with one or more spontaneous mammary gland tumours that were treated by surgical excision, were included in the study. The primary clinicians that saw these cases kindly provided the patient records and the surgically excised mammary tumours collected in 10% neutral buffered formalin. Full owner consent was obtained before sample collection.

The following information was determined from the patient records: primary complaint, breed, age, body condition score, age at neutering, parity, general clinical exam findings, and information regarding any prior investigations related to the presenting mammary gland tumours. According to the tumour diameter specified in the patient records,

tumours were classified as T1 (≤ 3 cm in greatest dimension), T2 (tumour > 3 cm but ≤ 5 cm in greatest diameter), or T3 (tumour > 5 cm in greatest diameter), following the World Health Organization guidelines as applied by Sorenmo et al. (2009).[29] Other gross pathological features of the tumours including adherence of tumour mass to the underlying tissues and ulcerations of the skin overlying the tumour, were also extracted from the patient records.

2.2.2 Histopathology and tumour classification

The formalin-fixed mammary gland tumours received from the primary clinicians were further processed in the histopathology laboratory of the VTH. Briefly, tumours were dehydrated in a gradient of alcohol, embedded in wax, processed into thin sections (3 µm), stained with haematoxylin and eosin, and examined microscopically. When multiple tumours were present in a single dog, only the tumour with the greatest diameter was considered for histological examination. Immunohistochemistry for α-smooth muscle actin (SMA) was performed to evaluate a myoepithelial origin of the neoplastic cells using an anti-α-SMA antibody (Sigma-Aldrich, St. Louis, MO, USA), following the standard protocols using vascular smooth muscles as the positive control and cardiac muscle as the negative control. Mammary tumour classification was performed according to the definitions in the 2011 classification proposed by Goldschmidt and colleagues.[14] Histological malignancy of the tumours was determined following the criteria described in the 2011 Goldschmidt classification: tumour type, nuclear and cellular pleomorphism, mitotic index, presence of randomly distributed areas of necrosis within the neoplasm, peri-tumoural and lymphatic invasion, and the presence of intra-tumoural inflammatory cell infiltration.[14] All carcinomas except inflammatory carcinomas were graded according to the guidelines provided by Pena et al. (2013).[19] Briefly, tubule formation, nuclear pleomorphism, and mitotic counts were considered and rated on a scale from 1 to 3. The scores for each category were added together and the total scores were used to determine the histological grades for each tumour. In heterogeneous carcinomas, tubular scoring was assessed in the most representative malignant area. In complex and mixed tumours, the percentage of tubular

formation was scored considering only epithelial areas, and nuclear pleomorphism was evaluated in all the malignant components.

2.2.3 Statistical analysis

Chi-squared test was performed to determine whether the malignant mammary tumours were equally distributed among the thoracic, abdominal and inguinal mammary glands. Chi-squared test was followed by the Marascuillo process to identify the glands which had a significantly different proportion of malignant mammary tumours compared to the other glands. For this, the number of malignant mammary gland tumours in each anatomical location was compared with the mammary gland tumours in other locations in a pair-wise manner using a calculated absolute and critical value for each pair. The difference was considered significant if the calculated absolute value was greater than the critical value. The same statistical methods were used to investigate differences in the distribution of malignant mammary neoplasms among T1, T2 and T3 tumour size categories. Single proportion test was used to compare the malignant tumour proportions in the right and left inguinal mammary glands, respectively. All statistical tests were performed using add-on for Excel/XLSTAT software (Version 2017.4) (Addinsoft, New York, NY, USA). Differences were considered significant if the calculated p values were < 0.05.

2.3 Results

2.3.1 Clinical characterization of dogs with mammary gland tumours

Seventy-four dogs with mammary gland tumours were included in the study. Thirty-six (48.6%) of them were from Veterinary Teaching Hospital (VTH), Peradeniya, and 38 (51.4%) were from the veterinary practice in Colombo (VPC).

Out of the 74 dogs included in the study, 41 (55.4%) dogs were presented primarily seeking veterinary care for mammary neoplasia. Among these, 11 dogs had tumours that had been

previously diagnosed as benign mammary neoplasms by cytology. In addition to the mammary neoplasm, reduced appetite and lethargy were secondary complaints in 10 (13.5%) and 17 (23.0%) dogs, respectively. In 33 (44.6%) dogs, mammary neoplasia was detected during a clinical examination when the dog was presented for an unrelated complaint. These dogs were presented for veterinary care primarily due to reduced appetite (n = 19, 25.7%) or lethargy (n = 14, 18.9%). Interestingly, the owners of 9 (12.1%) of the dogs admitted that even though they had noticed the mammary masses in their dogs prior to the clinical exams, they did not seek veterinary care specifically regarding them, assuming that the masses were harmless.

During clinical examination of the 74 dogs, lymphadenopathy was identified in 40 (54.1%) dogs. According to the clinical records, lymphadenopathy was detected in inguinal lymph nodes alone in 10 (13.5%) dogs, while both inguinal and popliteal lymph nodes were concurrently enlarged in 15 (20.3%) dogs. The affected lymph nodes were not specified in 15 (20.3%) dogs. Cytological examination of fine needle aspirates from the enlarged lymph nodes was performed in only 4 dogs and none of the aspirates were reported to contain neoplastic cells. Further, concurrent with lymphadenopathy, dyspnoea or pyrexia was detected during clinical examination in 16 (21.6%) and 14 (18.9%) dogs, respectively. Lateral thoracic radiographs had been taken in six dogs and evidence suggestive of pulmonary tumour metastasis was observed in 3 dogs. Weight loss was recorded in 7 out of the 30 dogs for which previous weight records were available. Two dogs were diagnosed with pyometra in addition to mammary neoplasia. Overall, 54 dogs, which accounted for approximately 75% of the group, were systemically ill at the time of presentation.

Thirty nine of the 74 dogs presented were crossbred dogs which represented approximately half of the group (50%). German shepherd was the most common pure dog breed (n = 21, 28.4%), followed by dachshund (n = 2, 2.7%). Single cases of following breeds were also observed: Boxer, Fox Terrier, Japanese Spitz, Pomeranian, Tibetan Terrier, Cocker Spaniel, English Springer Spaniel, Pekingese, Doberman, Dalmatian, Great Dane, Labrador Retriever, Rottweiler, and Rhodesian Ridgeback.

The overall mean and median ages of dogs with MGTs were 8.0 (SD 2.4) years and 8.0 years, respectively. The age of the dogs with MGTs was further analysed separately in 4 categories, namely: 0–4 years, 5–8 years, 9–12 years and ≥ 13 years. The most frequently represented age category was 5–8 years, which included 41 (55.4%) dogs. There were 26 (35.1%) dogs in the 9–12 years category. The categories 0–4 years and ≥13 years, which included the youngest and oldest dogs, were less frequently represented as there were only 4 (5.4%) and 3 (4.0%) dogs in those categories, respectively. The mean age of the dogs with benign and malignant MGTs were 6.7 (SD 1.66) years and 7.9 (SD 2.15) years, respectively. There was no significant difference between the age of the dogs with benign tumours and malignant tumours.

Primary clinicians had used the 1–5 body condition scoring system (BCS) described by Eastland-Jones et al. (2014).[11] Accordingly, the body condition of the majority of dogs was BCS 3 with 42 (56.8%) dogs in this category. BCS 2 and BCS 4 categories included 15 (20.6%) and 13 (17.6%) dogs respectively, while the BCS 5 group was the least represented group of all (n = 4, 5.4%). Reproductive status of the dogs with MGTs is summarized in Table 1. The majority of dogs were intact, while the remainder had been spayed at varying ages.

Table 2.1 Reproductive status of the dogs with mammary gland tumours

Reproductive status	# of cases	%
Intact	46	62.2
OHE: ≤ 3 years	8	10.8
OHE: 4 - 6 years	12	16.2
OHE: ≥ 7 years	6	8.1
Unknown	2	2.7

2.3.2 Gross pathological characterisation of canine mammary gland tumours

Tumours were detected in a single mammary gland in 53 (71.6%) dogs, while 21 (28.3%) dogs had tumours in multiple mammary glands. The distribution of the mammary tumours

among the mammary glands is summarised in Table 2.2. Inguinal mammary glands were most often affected: 46 (62.1%) dogs had tumours in these glands. Surprisingly, a significantly higher ($p < 0.001$) number of dogs had tumours in the left inguinal gland (n = 42, 56.8%) than the right (n = 4, 5.4%). No such significance was detected in mammary gland tumours in the thoracic or abdominal mammary glands. Nine dogs with inguinal mammary tumours had another mammary tumour in a different location, while four dogs with inguinal mammary gland tumours had multiple tumours in other glands.

Table 2.2 Distribution of mammary gland tumours.

For malignant neoplasms, different superscripts (a, b) between different tumour locations indicate significant differences. Critical and absolute values: Thoracic—Abdominal (0.13, 0.04), Thoracic—Inguinal (0.17, 0.55), Abdominal—Inguinal (0.17, 0.50).

Location	Benign	Malignant	Total
Thoracic	5 (55.6%)	9 (13.8%)[a]	14 (18.9%)
Abdominal	2 (22.2%)	12 (18.4%)[b]	14 (18.9%)
Inguinal	2 (22.2%)	44 (67.7%)[b]	46 (62.2%)
Total	9 (100%)	65 (100%)	74 (100%)

The size of the mammary gland tumours is summarized in Table 2.3. Overall, 31 dogs had T2 tumours, 25 had T3 tumours, and 18 had T1 tumours. Dogs which were primarily presented for mammary neoplasia had either T2 (n = 18) or T3 (n = 25) tumours, but none had T1 tumours. The MGTs which were incidentally detected in a clinical exam of a dog that was presented for unrelated complaints were either T1 (n = 18) or T2 (n = 15).

The size of the mammary gland tumours is summarised in Table 2.3. Overall, 31 dogs had T2 tumours, 25 had T3 tumours, and 18 had T1 tumours. Dogs which were primarily presented for mammary neoplasia had either T2 (n = 18) or T3 (n = 25) tumours, but none had T1 tumours. The MGTs which were incidentally detected in a clinical exam of a dog that was presented for unrelated complaints were either T1 (n = 18) or T2 (n = 15).

Table 2.3 Size of canine mammary gland tumours.

For malignant neoplasms, different superscripts (a, b) between different tumour size categories indicate significant differences. Critical and absolute values: T1–T2 (0.18, 0.32), T1–T3 (0.17, 0.22), T2–T3 (0.2, 0.1).

	Benign	Malignant	Total
T1	8 (88.8%)	10 (15.4%)[a]	18 (24.3%)
T2	1 (11.1%)	30 (47.7%)[b]	31 (41.9%)
T3	0 (0%)	25 (38.5%)[b]	25 (33.8%)
Total	9 (100%)	65 (100%)	74 (100%)

Ulceration of the skin overlying the tumour was observed in 20 (27.0%) dogs, while 15 (20.3%) tumours were fixed to the underlying tissues.

2.3.3 Histological classification of mammary gland tumours

Sixty-five (87.8%) of the tumours examined histologically were classified as malignant, while 9 (12.2%) were classified as benign. All mammary gland tumours histologically identified as malignant included at least three cellular or nuclear criteria of malignancy. Histological evidence of peri-tumoural (n = 6) and lymphatic (n = 4) invasion was identified in 10 malignant tumours, while randomly distributed areas of necrosis within the neoplasm were observed in 16 malignant tumours.

Using the histologic classification of malignant mammary gland tumours, tumours were identified in three categories: carcinomas, carcinomas-special types, and sarcomas (Table 2.4). There were 9 sub-types of carcinomas, 6 sub-types of special carcinomas and 3 sub-types of sarcomas. Of the carcinoma sub-types, simple carcinoma (n = 13, 17.6%) and mixed-type carcinoma (n = 10, 10.8%), were the most frequent sub-types in the carcinoma group. Thirteen simple carcinomas included 5 tubular carcinomas, 7 tubulo-papillary carcinomas, and 1 cribriform carcinoma. In addition, single cases of ductal carcinoma,

anaplastic carcinoma, and carcinoma-spindle cell variant were identified in the carcinoma group.

Adenosquamous carcinoma (n = 8, 10.5%) was the most frequent special type carcinoma, while hemangiosarcoma (n = 2, 2.7%) was the most frequent type of sarcoma. Simple adenoma (n = 3, 4.0%) was the most frequent benign MGT sub-type, while single cases of fibroadenoma and mixed benign tumour sub-types were observed. Immunostaining using antibodies against smooth muscle actin (α-SMA) was present in 3 MGTs interpreted to be complex carcinomas and in 1 neoplasm that was classified as a malignant myoepithelioma. The absence of immunostaining was used to support a classification of mammary gland fibrosarcoma in one case.

Intra-tumoural inflammatory cell infiltrates were observed in 21 malignant tumours; moderate intra-tumoural cell infiltration was identified in 11 tumours, and it was low and marked in 6 and 4 tumours respectively. Sixty carcinomas were graded. Of the sixty, 24 were classified as grade I, 19 were grade II, and 17 were grade III carcinomas.

All the dogs that were presented primarily due to mammary neoplasia had neoplasms that were classified as malignant, and all the benign tumours included in this study had been detected incidentally during clinical examination. No clinical features allowed definitive determination between malignant and benign neoplasms. However, ability to associate malignancy with a clinical feature in this study was impaired by the small number of dogs with dogs with benign mammary neoplasms. In all the dogs with multiple mammary tumours, the tumours with the greatest diameters were malignant. In addition, all the tumours with surface skin ulcerations, and those which were fixed to the underlying tissues, were malignant.

The tumour location was predictive of malignancy with a significantly higher proportion of malignant mammary gland tumours developing in the inguinal mammary glands than in the thoracic and abdominal mammary glands (Table 2.2). Regarding the tumour size, the

proportions of malignant T2 or T3 tumours were significantly higher compared to the proportion of malignant T1 tumours (Table 2.3). Thus, T2 or T3 tumours were more likely to be malignant than T1 tumours. However, there was no significant difference between the proportions of malignant T2 and T3 tumours. This indicates that the further differentiation of large mammary tumours in to T2 or T3 categories does not provide any additional advantage when predicting malignancy.

Table 2.4 Histological classification of canine mammary gland tumours

Malignant tumours	# of cases	%
Carcinoma	45	
Carcinoma: simple	13	17.5
Carcinoma: mixed	10	13.5
Carcinoma: solid	6	8.1
Intra-ductal papillary carcinoma	5	6.8
Comedocarcinoma	5	6.7
Carcinoma: complex	3	4.1
Ductal carcinoma	1	1.3
Carcinoma: anaplastic	1	1.3
Carcinoma *in situ*	1	1.3
Carcinoma special types	16	
Adenosquamous carcinoma	8	10.8
Squamous cell carcinoma	3	4.1
Lipid-rich carcinoma	2	2.7
Carcinoma: spindle cell variant	1	1.3
Inflammatory carcinoma	1	1.3
Malignant myoepithelioma	1	1.3
Sarcoma	4	
Haemangiosarcoma	2	2.7
Fibrosarcoma	1	1.3
Osteosarcoma	1	1.3
Benign tumours	9	
Complex adenoma	5	6.7
Simple adenoma	3	4.0
Ductal adenoma	1	1.3

2.4 Discussion

The present study reports a systematic evaluation of the clinicopathological features of mammary gland tumours in 74 Sri Lankan dogs. In these dogs, 88% of the mammary tumours were histologically classified as malignant. This proportion of is higher than the 40–50% of CMGTs reported to be malignant in studies conducted in United States of America (USA),[1] Canada,[18] Japan,[35] and Mexico.[23] However, the proportion of malignant tumours observed in Sri Lankan dogs was similar to the rates reported from India (83%) and Brazil (86%).[9,34] The reasons for the higher proportion of malignant mammary tumours in dogs in Sri Lanka, Brazil and India are unknown. It is possible that the malignant mammary tumours were overrepresented in these countries as a consequence of frequent exposure of the dogs to carcinogens which may not be present in countries such as the USA, Canada, Japan and Mexico. However, it is also possible that malignant mammary neoplasms were over-represented in the developing countries because of under detection of benign mammary gland tumours. Benign CMGTs are often incidental clinical exam findings, rather than the primary concerns of the dog owners.[21] The incidental detection of benign CMGTs during a clinical examination may be less likely to occur in developing countries because dog owners in these countries may seek veterinary care less frequently compared to owners in more developed countries. It appears likely that owners may not seek veterinary advice unless they observe a rapidly growing ulcerated mammary gland mass and such masses are more likely to be malignant tumours.[22] This is supported by the observation in the present study that all dogs that presented for a mammary gland mass had malignant tumours, while all the benign tumours observed in this study were from dogs that had presented to the veterinarian for reasons other than the mammary neoplasia. The high percentage of CMGTs that were malignant at the time of presentation in Sri Lanka suggests that veterinarians should be aware of this disease and consider mammary neoplasia as a serious health problem among Sri Lankan dogs. In this study, when there were multiple mammary tumours in a single dog, only the tumour with the greatest diameter was considered for histological analysis due to the financial limitations. The preferential examination of the largest neoplasm could also have contributed to the higher proportion of CMGTs being malignant in this study.

Nine malignant CMGTs included in the present study had been previously diagnosed as benign, using cytology. This discrepancy could be either due to the limited capability of cytology to differentiate benign from malignant tumours[1] or possible benign to malignant transformation which had occurred during the time between the initial diagnosis and the second examination.[22,31] This observation suggests that pathologists should be cautious when classifying a mammary gland tumour as benign solely on cytology. Additionally, it suggests that benign tumours should be carefully monitored for evidence of progression to a malignant neoplasm. It is noteworthy that nine dog owners who had observed the CMGTs in their dogs had not considered them as conditions requiring veterinary care. This indicates that some Sri Lankan dog owners may not to be sufficiently aware of the adverse consequences of CMGTs and emphasises the necessity of improving awareness on mammary neoplasms among the Sri Lankan dog owners.

A majority of affected dogs in the present study were 5–8 years old, and the mean age at the diagnosis of mammary neoplasia was 8.0 (SD 2.47) years. The mean age reported from Sri Lanka is comparatively lower than the age of diagnosis of mammary neoplasia in dogs reported from Sweden (9.33 years),[16] Slovenia (10 years),[4] Turkey (10.3 years),[28] Canada (11 years),[18] Brazil (11.6 years and 12 years),[8,13] Mexico (9–12 years)[23] and Czech Republic (13 years).[36] However, the reported ages of diagnosis of mammary neoplasia in three studies from India, Bhutan and Malaysia were 7–9 years,[15] 8.4 years,[5] and 8.6 years[22] respectively. It is interesting to note that dogs from Asia have been reported to develop MGTs at an earlier age than dogs from North America, South America, and Europe. Whether this reflects exposure to an external carcinogen, or a genetic predisposition in dogs in Asian counties is unknown. Other possible factors include different feeding practices, spaying practices, and immunisation protocols followed in Asian countries compared to those of North American, South American and European countries.[25] Alternatively, it is possible that dogs in the Asian countries do not live long and the earlier onset of mammary neoplasia detected in these countries is simply due to fewer old dogs in these populations.

In the present study, the majority of dogs with CMGTs were crossbred dogs. Generally, crossbred dogs are considered to be comparatively less predisposed to mammary gland tumours compared to pure breeds.[36] In Sri Lanka, crossbred dogs are reportedly the most common pet dog breed.[15] Thus, the predominance of them in the present study is most likely a reflection of their commonality, rather than a true breed predisposition. Similarly, the over-representation of German shepherds in the present study might also be due to their high popularity in Sri Lanka and may not necessarily indicate a breed predisposition. The minimal representation of Labradors in this study is noteworthy, considering the reportedly high popularity of this breed among Sri Lankan dog owners.[25]

An interesting feature regarding the reproductive histories of the dogs in the present study was that over half of the dogs were nulliparous. A recent study conducted in Switzerland in 2018 suggested that nulliparous dogs are at a significantly higher risk for developing mammary tumours compared to multiparous dogs.[33] The elevated risk was attributed to the higher frequency of pseudopregnancy and oestrus in nulliparous dogs than multiparous dogs. As both pseudopregnancy and oestrus increase the production of female reproductive hormones, the mammary gland tissues of nulliparous dogs may be exposed to greater amounts of female reproductive hormones than the mammary gland tissues of multiparous dogs.[33] While the results of the present study suggest that nulliparous dogs in Sri Lanka may similarly be at higher risk for CMGTs, it has to be noted that the proportion of dogs in the studied populations that are nulliparous is unknown. Therefore, it is possible that the high proportion of dogs with CMGTs that were nulliparous in the study was simply due to the high proportion of dogs in Sri Lanka that are nulliparous.

Most of the dogs in the present study had an ideal body condition score (BCS 3). Obesity at 1 year of age and in the year prior to diagnosis of mammary gland neoplasia is significantly associated with a higher risk of CMGTs.[1] In contrast, we found only 5 obese dogs in our study. While this may suggest obesity does not predispose to CMGTs, this cannot be determined without the knowledge of overall proportion of obese dogs in the population.

The proportion of dogs with multiple mammary tumours in this study (27.6%) was higher than the proportions of dogs reported in the majority of previous studies, which are mainly of dogs from Western countries.[15,26] An exception was a Malaysian study in which 29.2% of dogs were reported to have multiple CMGTs.[22] The occurrence of multiple mammary gland tumours is well recognised in dogs and is believed to be due to the concurrent exposure of all mammary glands in a single animal to circulating reproductive hormones.[31] The resulting tumours are more likely to be at the same stage of development and are more frequently benign than malignant.[31] However, benign to malignant transformation may occur in some tumours over time, resulting a combination of malignant and benign tumours in the same animal.[31]

In the present study, the majority of CMGTs were detected in the inguinal mammary glands. This distribution is consistent with the findings of many previous studies.[27,28,4] The frequent involvement of the inguinal glands is attributed to their abundant tissue mass and prolonged secretory activity, compared to other glands.[22] However, unlike in previous studies, our results show significantly higher involvement of the left inguinal gland compared to the right. As both left and right inguinal glands have been previously reported to be affected at equal rates,[22,23] the marked left gland involvement observed in the present study is difficult to explain. In addition to a significantly greater number of CMGTs in the inguinal mammary glands, the tumours that did occur in this location were malignant. This has not been described in previous studies, and the reason for a higher proportion of malignant inguinal CMGTs in the present study is unknown.

The results of the present study suggest that tumours which have a diameter > 3 cm are more likely to be malignant. This is consistent with the findings of previous studies. A retrospective study conducted by Philibert and colleagues (2003)[20] showed that dogs with tumours > 3 cm in diameter have decreased overall survival compared to the dogs with tumours < 3 cm in diameter. In another study, tumour size of > 3 cm diameter was correlated with several factors indicating poor prognosis, such as loss of hormone receptors and higher proliferation index.[29] However, tumour size alone does not confirm

the malignancy of a mammary tumour, and histological examination is essential for confirmation. In the present study, all the tumours with surface skin ulceration, and tumours which were fixed to the underlying tissues, were malignant, indicating that these features could also be predictive of malignancy. Previous studies also indicate that malignant mammary gland tumours which are usually large in size are more likely to develop ulceration, due to more frequent contact with rough surfaces compared to small sized benign tumours.[22] Since malignant neoplasms invade or infiltrate surrounding muscle, nerve, blood vessels, and connective tissues, they are also more likely to become fixed to the underlying tissues.[22]

The histological diversity of the malignant CMGTs included in the present study was high. In fact, out of the 23 malignant CMGT sub-types listed in the Goldschmidt classification, 18 were reported in the present study. A recent prospective study conducted in Italy confirmed the prognostic significance of the Goldschmidt classification.[21] Given the high histological diversity revealed by the present results, Sri Lankan pathologists should be able to accurately differentiate tumour sub-types in order to provide reliable prognostic information. In this paper we used α-SMA to help differentiate between complex and simple carcinomas. In addition, the same antibody was used to help differentiate the myoepithelial origin of a malignant myoepithelioma, and to exclude myoepithelial origin within a mammary gland fibrosarcoma. However, definitive differentiation of myoepithelial cells not possible using α-SMA alone. Instead, it is currently recommended that p63 or a panel of antibodies be used to differentiate between these tumour types. In the present study, only α-SMA was used to reproduce the likely situation in developing countries in which p63 is not often available, and clients are unlikely to be able to afford a panel of immunostains. Interestingly, one of the simple mammary carcinomas reported in the present study co-existed with a cutaneous mast cell tumour. Therefore, apart from being classified as a mammary carcinoma, it was identified as a collision tumour referring to the mixed presentation. Collision tumours are a type of a mixed tumour with 2 foci of neoplasia which develop adjacent to one another yet remain separate.[24] These tumours are rare, and there is minimal information regarding treatment recommendations and outcome for animals.[24]

Previous studies have reported that dogs with mammary gland tumours are generally healthy at the initial presentation.[21] However, most of the dogs included in our study were systemically ill at the time of presentation, which might be due to tumour metastasis or other concurrent diseases. Systemic illness due to tumour metastasis was confirmed only in a few cases in the present study, due to the unavailability of necessary diagnostic testing. In systemically ill dogs, pre-surgical patient stabilisation is important for successful surgical and post-surgical management.[31] Therefore, these results suggest that Sri Lankan veterinary surgeons should be vigilant about the pre-surgical patient stabilisation procedures to minimise possible post-surgical complications.

In summary, a main finding from this study was a high proportion of malignant CMGTs with a wide variety of histological sub-types. The typical presentation was intact, middle-aged mixed breed dogs. This is broadly similar to what has been reported in India and Brazil, however notable differences were a younger age at diagnosis and lower body condition score of the dogs with mammary tumours. The reasons for these differences are not known but may be due to differences in risk factors, differences in the age and breed of the dog population or different behaviours of owners regarding the frequency seek veterinary advice. Further investigation is needed to better understand the above possibilities to improve the treatment and prevention of CMGTs in dogs in Sri Lanka.

2.5 Bibliography

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Chapter 3 : Mammary gland disease in dogs in New Zealand

3.1 Introduction

Outcome known canine mammary gland tumour (CMGT) cases from New Zealand (NZ) were used for the inflammation-related prognostic marker studies included in this thesis. The NZ cases were used instead of the Sri Lankan cases described in the previous chapter as the Sri Lankan cases lacked sufficient follow-up data. The New Zealand CMGT cases were identified by searching the surgical biopsy database of a commercial veterinary diagnostic laboratory: IDEXX Laboratories, NZ. In addition to identifying outcome known cases for prognostic marker studies, the IDEXX database search generated a large amount of information regarding mammary gland diseases in NZ dogs. As no previous studies have investigated mammary gland diseases in dogs in NZ, the information gathered from the IDEXX database search was used to identify clinicopathological features of mammary gland diseases in dogs in NZ. This chapter is a comprehensive description and analysis of the compiled data from the IDEXX database. Additionally, it was determined whether the age, breed or reproductive status of the dog or the size of the mammary mass was associated with the subsequent histological diagnosis made by the pathologist.

Female dogs often develop mammary gland disease (MGD) including neoplastic and non-neoplastic conditions. Histopathology is considered as the gold standard to diagnose MGDs in dogs[12] and biopsies are frequently submitted to diagnostic laboratories for histopathological examination. Studies performed in other countries have indicated that, among the laboratory submissions of mammary gland biopsies, neoplastic disease is more common than non-neoplastic disease.[20,7] Of the mammary gland neoplasms, the proportion of mammary gland neoplasms that are malignant ranges from 50 to 90%.[20] Currently, there are no studies evaluating submissions of MGDs to NZ diagnostic laboratories. Therefore, the primary aim of this study was to determine the proportion of canine MGD submissions to NZ diagnostic laboratories that are diagnosed as neoplastic

disease and, of the mammary gland neoplasms, what proportion are classified histologically as malignant.

Currently no clinical features were shown to predict whether a canine mammary gland mass is more likely to be non-neoplastic or neoplastic. Likewise, of the masses that are neoplastic, only size is recognised to predict the histological diagnosis with most studies reporting that larger masses are more likely to be histologically-malignant than smaller ones.[10,19] The secondary aim of this study was to determine if the age, breed or reproductive status of the dog or the size of the mammary mass was associated with the subsequent histological diagnosis made by the pathologist.

While multiple studies have evaluated MGDs in dogs elsewhere in the world, the age at ovariohysterectomy (OHE), level of veterinary care, and willingness of the veterinarians to submit diagnostic samples contribute to the significant differences in the MGDs seen between different countries.[2,23,3] Therefore, while MGDs in dogs in NZ are expected to be similar to countries where similar de-sexing practices and socioeconomic conditions exist, it was considered possible that diagnoses in NZ could show differences from those reported in other countries. Although only approximately representative of the general population of female dogs in NZ, the present study provides some useful insights on the commonly diagnosed MGDs in a relatively large group of dogs. Further, it allows comparison of MGDs in dogs in NZ to similar laboratory-based, retrospective studies of MGDs conducted elsewhere in the world.

3.2 Material and methods

2.2.1. Data collection and categorisation

This retrospective study included canine mammary gland samples submitted to IDEXX Laboratories, NZ, between 2012 and 2016. The cases included in the study were identified by a search carried out in the IDEXX surgical biopsy database using the following key word combinations: Dog + mammary gland, Dog + mammary mass, Dog + mammary tumour. Male dogs which had mammary lesions were excluded, as were dogs which had lesions

originating from tissues adjacent to the mammary gland rather than the mammary gland itself. For each identified case, the age, reproductive status, and breed of the dog, along with the number of mammary lesions, were derived from the submission form that accompanied the lesion. Tumour size was identified from the pathology report; the greatest diameter of all tumours was measured using a ruler during trimming of the sample by the pathologist. The histological diagnosis was also recorded from the pathology report, including tumour histological sub-type and the presence of intra-vascular or intra-lymphatic tumour emboli where appropriate. The criteria and classification system used to determine tumour diagnosis was at the discretion of the pathologist who read the case, as cases were not available for re-examination.

3.2.2 Statistical analyses

Independent sample t-test or Chi-squared test of independence were performed to investigate differences in age, breed, reproductive status, or size of the mammary mass between non-neoplastic and neoplastic lesions, and between neoplasms histologically classified as benign versus those classified as malignant. All statistical analyses were performed using SPSS Version 25 (IBM Corporation, Armonk, New York) and p values < 0.05 were considered to indicate significant differences or associations.

3.3 Results

3.3.1 Selected cases

The present study included samples taken from 798 dogs with MGDs. Among them there were 459 (55.5%) intact dogs and 339 (45.5%) spayed dogs. The mean age of the dogs was 8.1 years (SD 2.7). The dogs included 78 different breeds with 672 (84.2%) purebred and 126 (15.8%) cross-bred dogs.

Histological examination of the masses had been performed by one of the seven veterinary pathologists who worked at the laboratory during the period of the study. The precise

criteria that were used by each pathologist to differentiate between a benign and malignant neoplasm were not recorded and may have varied between individual pathologists. Around two-third of the neoplasms were sub-classified according to the criteria reported by Goldschmidt et al. (2011) while the classification system used for the other neoplasms was not recorded.

3.3.2 Types of mammary gland lesions

Neoplastic lesions

Neoplasia was diagnosed in 674 (84.5%) dogs with MGD. These mammary gland tumours were present as solitary masses in 592 (87.8%) dogs while 82 (12.2%) dogs had multiple tumours (Table 3.1). Due to the submission of multiple tumours from some dogs, a total of 772 individual mammary gland neoplasms were included in the study. Of these 772 neoplasms, 340 (44.1%) were classified using histopathology as benign while 432 (55.9%) were classified as malignant.

Of the 772 mammary neoplasms, 701 (90.8%) were derived from mammary gland epithelium while 71 (9.2%) were derived from mammary gland tissues other than mammary epithelium. A variety of histological sub-types were included among the neoplasms derived from the mammary epithelium with mixed mammary tumour and simple carcinoma being the most common sub-types in benign and malignant categories respectively (Table 3.2). All the 71 neoplasms which had originated from tissues in the mammary gland other than mammary epithelium were solitary neoplasms and the majority were histologically classified as malignant. The most common histological sub-type was sarcoma although a variety of other types were diagnosed.

Intra-lymphatic or intra-vascular tumour emboli were observed in 72 (16.7%) of the 432 malignant mammary neoplasms including 65 (17.4%) of the 374 malignant mammary epithelial neoplasms. Emboli were visible in 7 (12.1%) of the 58 malignant neoplasms that originated from other mammary gland tissues including four squamous cell carcinomas, two mast cell tumours and one pleomorphic sarcoma. Regional lymph nodes were

submitted for histology from 47 (7%) of the dogs with neoplastic MGD cases and neoplastic cells were visible in 21 of them. These 21 cases included nine neoplasms in which lympho-vascular invasion had been noted in the descriptions of the primary tumour.

Non-neoplastic lesions

Of the 798 dogs included in the study, a diagnosis of a non-neoplastic MGD was made in 124 (15.5%) dogs. Multiple non-neoplastic mammary lesions were diagnosed in 35 dogs. Of the reported non-neoplastic conditions, the most common were dysplastic or hyperplastic conditions of the mammary gland epithelium (78 ,62.9%) including ductal hyperplasia, ductal ectasia, lobular hyperplasia or adenosis and fibroadenomatous changes. Other common conditions included fibrosclerosis (28, 22.6%), cysts (15,12.1%), and mastitis (16, 12.9%). Cholesterol granulomas, mammary abscessation and foreign body granulomas were also rarely reported.

3.3.3 Age, reproductive status, and breed of dogs with mammary gland diseases

Dogs with non-neoplastic versus neoplastic lesions

The mean age of the dogs with neoplastic lesions was 8.7 years (SD 2.1) which was not significantly different from that of dogs with non-neoplastic lesions (8.6 years, SD 2.5, p = 0.09, Independent sample t-test). Of the 339 dogs that were spayed, 285 (84%) had neoplastic lesions which was not significantly different from the proportion of intact dogs that had neoplastic MGDs (389, 84.7%, p = 0.79, Chi-squared test). Neoplastic MGD was diagnosed in 613 of 672 (90.9%) dogs classified as purebred which was significantly higher than the 61 of 126 (48.4%) crossbred dogs that were diagnosed with neoplastic MGD (p < 0.001, Chi-squared test). The most frequently reported dog breeds with mammary gland neoplasia were Labrador Retriever (50, 7.4%), Jack Russell Terrier (37, 5.5%), Border Collie (35, 5.1%), German Shorthaired Pointer (34, 5.1%) and Huntaway (34, 5.1%).

Dogs with benign versus malignant neoplastic lesions

The mean age of the dogs with malignant mammary neoplasms was 8.9 years (SD 2.4) which was not significantly different from the mean age of dogs with benign neoplasms (8.2 years, SD 2.7, $p = 0.15$, Independent sample t-test). The reproductive status of the dogs was also not significantly associated with whether the neoplasm was benign or malignant ($p = 0.36$, Chi-squared test).

3.3.4 Size of the mammary gland lesions

The mean diameter of the neoplastic mammary lesions was 3.0 cm (SD 2.3) which was not significantly different from mammary lesions histologically classified as non-neoplastic (2.8, SD 1.3 cm, $p = 0.21$, Independent sample t-test). However, for the neoplastic lesions, mammary neoplasms histologically classified as benign were significantly smaller (1.2 cm, SD 2.8) than the neoplasms classified as malignant (3.2 cm SD 2.2; $p = 0.01$, Independent sample t-test).

Table 3.1 Canine mammary gland biopsies submitted to IDEXX laboratories, NZ from 2012-2016.

**Refers to number of dogs and †refers to number of lesions.*

	N	%
Type of lesion (n* = 798)		
Neoplastic	674	84.5
Non-neoplastic	124	15.5
Number of tumours in an individual dog (n* = 674)		
Single	592	87.8
Multiple	82	12.2
Two	67	
Three	14	
Four	1	
Histological malignancy status (n † = 772)		
Benign	340	44.1
Malignant	432	55.9
Histological type (n † = 772)		
Considered to be derived from mammary gland epithelium	701	90.8
Derived from other tissues in the mammary gland	71	9.2
Size of non-neoplastic lesions (n† = 124)		
< 3cm	75	60.4
3-5 cm	39	31.5
> 5cm	10	8.1
Tumour size (n † = 772)		
< 3cm	370	47.9
3-5 cm	210	27.2
> 5cm	192	24.9
Age (n* =798)		
< 5 years	67	8.4
5-10 years	523	65.6
> 10 years	186	23.3
Unknown	22	2.7
Breed (n* = 798)		
Pure-bred	672	84.2
Cross-bred	126	15.8
Sex (n* = 798)		
Intact	459	57.5
Spayed	339	42.5

Table 3.2 Histological sub-types of neoplastic mammary gland lesions

	N	%
Tumours originated from mammary epithelium		
Benign	**327**	
Mixed mammary tumour	128	39.1
Complex adenoma	105	32.1
Intra-ductal adenoma	23	7.0
Ductal adenoma	22	6.7
Simple adenoma	33	10.1
Cyst adenoma	13	4.0
Papillary adenoma	1	0.3
Myoepithelioma	1	0.3
Fibropapilloma	1	0.3
Malignant	**374**	
Simple carcinoma	160	42.8
Complex carcinoma	54	14.4
Ductal carcinoma	32	8.6
Intra-ductal papillary carcinoma	30	8.0
Anaplastic	25	6.7
Solid carcinoma	20	5.3
Mixed mammary carcinoma	16	4.3
Carcinoma arising in a mixed mammary tumour	14	3.7
Carcinosarcoma	11	2.9
Malignant myoepithelioma	4	1.1
Inflammatory carcinoma	3	0.8
Carcinoma and malignant myoepithelioma	1	0.3
malignant myoepithelioma arising in a mixed mammary tumour	1	0.3
Mucinous carcinoma	1	0.3
In situ carcinoma	2	0.5
Tumours originated from tissues other than mammary epithelium	71	
Malignant	58	
Sarcoma	11	19.0
Haemangiosarcoma	8	13.8
Osteosarcoma	8	13.8
Chondrosarcoma	2	3.4
Liposarcoma	1	1.7
Leiyomayosarcoma	1	1.7
Pleomorphic sarcoma	1	1.7
Squamous cell carcinoma	23	39.7
Mast cell tumour	3	5.2
Benign	**13**	
Lipoma	11	84.6
Fibroma	1	7.7
Sebaceous adenoma	1	7.7

3.4 Discussion

The main aim of the present study was to determine which MGDs are diagnosed most frequently in diagnostic laboratories in NZ. The results revealed that the majority of mammary gland biopsies submitted for histopathology were diagnosed as neoplastic disease with slightly more neoplasms being classified as malignant than benign. Most of the neoplastic lesions were solitary with multiple neoplasm reported infrequently. A variety of histological sub-types were included both in benign and malignant neoplasm categories. The secondary aim of the study was to determine if the signalment of the dog or the size of the neoplasm could be used to predict the subsequent histological diagnosis. The results suggested that a mammary gland mass from purebred dog was more likely to be neoplastic than one from a crossbred dog and that malignant neoplasms were larger than benign ones. However, the size of the non-neoplastic lesions was not significantly different from the size of neoplasms and neither the age nor the reproductive status of the dog predicted whether a mammary gland mass was more likely to be neoplastic.

Most mammary gland biopsies submitted for histopathology to diagnostic laboratories in NZ were diagnosed as neoplastic disease. Furthermore, slightly over half of these neoplasms were classified histologically as malignant. The results of the present study are consistent with similar laboratory-based, retrospective studies conducted in Canada[15], Japan[28], and Mexico[20] which reported that 40—50% of the submitted neoplasms were malignant. However, the proportion of malignant neoplasms in the present study is much lower than the 80—88% of canine mammary gland tumours classified as malignant in similar studies of dogs from India[6], and Brazil[25]. The reasons for this difference are uncertain. It is possible that true differences in the rate of malignant neoplasms exist between the countries, potentially caused by a genetic predisposition for malignant neoplasms in dogs in some countries or exposure to a carcinogen that results in more frequent development of malignant neoplasms. Alternatively, the differences between countries may be artefactual, possibly because dogs in some countries only receive veterinary care when mammary gland masses are larger and so more likely to be malignant.

Only 12% of the dogs included in the present study had multiple neoplasms which was lower than the 60—70% reported by previous studies[11,4,22]. Multiple mammary neoplasms are suggested to develop due to simultaneous exposure of multiple mammary glands to reproductive hormones for a prolonged period.[5,27] Therefore, it is possible that more dogs in NZ are spayed earlier in life and avoid the prolonged exposure to reproductive hormones. However, due to the absence of statistics on the ages that dogs are spayed in NZ and elsewhere, it is difficult to determine if the age of spaying influences the development of multiple mammary masses. Alternatively, it is possible that some veterinarians may have submitted a single neoplasm for histopathology even when multiple masses were present. It is also possible that dog owners have declined surgical tumour excision or sample submission for histopathology from multiple tumour cases as they may have felt that multiple tumours were associated with a worse prognosis or if they were from old dogs and so were less willing to spend the money on histopathology.

A variety of histological sub-types were included in both in benign and malignant neoplasm categories. The mammary tumours included in the present study had been submitted between 2012-2016 and while most neoplasms were classified using the classification proposed by Goldschmidt et al. (2011)[12], some were likely to have been classified using older systems such as those by Hampe and Misdorp (1974)[13] or Misdrop et al. (1999).[14] The lack of consistent classification is a limitation of the study. Ideally, each diagnosis would have been confirmed and standardised by a single pathologist using the latest classification. However, older cases were not available for re-examination. While the lack of consistent classification may have introduced some error, it is interesting to note that the most common malignant and benign histological tumour sub-types identified in the present study were consistent with those reported in previous studies.[20,19,16,17] This consistency with other studies suggests that the neoplasms that developed in dogs in NZ are similar to those that develop in dogs elsewhere in the world.

A number of neoplasms of the mammary glands appeared to originate in tissues other than mammary gland epithelium. Such neoplasms were uncommonly reported in previous

studies[11,4] and it is possible that these neoplasms originated from surrounding tissue and subsequently invaded the mammary glands. Whether the submitters considered these primary mammary gland neoplasms or whether they simply developed in the mammary gland region could not be definitively determined.

A small number of inflammatory mammary carcinomas were diagnosed in the present study. As this particular subset of mammary neoplasm is a clinical diagnosis rather than a histological diagnosis[24], it appears likely that the clinical appearance of the neoplasm was reported to the pathologist and this allowed a diagnosis of inflammatory carcinoma in these cases.

Fibroadenomatous changes were reported in non-neoplastic lesions in the present study. Although these lesions were described in the Goldschmidt classification (2011)[12], there are no published reports on them suggesting this lesion is rarely diagnosed in other studies. It is uncertain why these were apparently common in the present study without the ability to re-examine the slides and confirm this diagnosis.

Of the malignant mammary neoplasms reviewed in the present study, intra-lymphatic or intra-vascular tumour emboli were reported in around 17% of cases. Currently, while histological criteria are used to classify mammary gland neoplasms as benign or malignant, it is unknown how many tumours that are classified as malignant will metastasise if not removed.[12] While the presence of tumour emboli does not guarantee that the neoplasm will metastasise, it strongly suggests the potential for a neoplasm to spread and eventually kill the host. Therefore, the findings of the present study emphasise the utility of histology in clinical decision making as it can highlight which mammary neoplasms need prompt attention. Additionally, the present results suggest the importance of submission of regional lymph nodes for histopathology as identification of neoplastic cells in the lymph node is also important for prognostic determination.

In this series of mammary gland biopsies, the size of the mass was not predictive of whether the mass was neoplastic or non-neoplastic, but malignant neoplasms in the study were significantly larger than benign ones. Malignant neoplasms were also larger than benign neoplasms in several previous studies.[18,21] Of the other clinical factors examined in the study, neither the age nor reproductive status of the dog was found to predict which masses were neoplastic or which neoplastic masses were malignant. A similar lack of association between age and benign or malignant status of a tumour was previously reported by Salas et al. (2015).[20] However, these results contrast with two other previous studies which reported that the mean age at diagnosis was lower for dogs with benign mammary neoplasms than dogs with malignant neoplasms.[22,26] Overall, the results of the present study confirm several previous studies that also reported that, for mammary masses, a presumptive diagnosis cannot be made from the clinical parameters and therefore histology is always recommended for diagnosis.[20,21]

In the present study, purebred dogs were significantly more likely to be diagnosed with neoplastic MGD than crossbred dogs. This is similar to previous studies[1,9,26] and could suggest a genetic predilection of purebred dogs for mammary neoplasms.[8] Alternatively, it is possible that purebred dogs are more likely to be used for breeding and so have been spayed later in life predisposing them to mammary neoplasia. However, it is possible the association was artefactual due to purebred dogs receiving more frequent veterinary care that resulted in the higher number of mammary gland lesions submitted to the diagnostic laboratories. The high number of mammary gland lesions observed in Labrador Retriever and Border Collie dogs in the present study may simply be due to the popularity of these breeds in NZ during the period that lesions were collected for the study.

A major limitation of this study is that it only included mammary masses submitted for histopathology and not all the mammary masses developed on dogs. Therefore, the results were confounded by the decisions made by veterinarian and owner whether or not to excise and submit a mammary mass for histopathology. Another limitation of the study

was the unavailability of some important clinical information regarding the mammary masses which was unable to be retrieved due to the retrospective nature of the study.

In conclusion, the present study included a large number of mammary gland lesions from dogs that were submitted for diagnostic evaluation. The majority of the masses were neoplastic with slightly more neoplasms classified as malignant than classified as benign. There were no clinical parameters that allowed a presumptive diagnosis suggesting that all mammary gland lesions in dogs should be considered potentially malignant and histology is necessary for diagnosis.

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Chapter 4 : Prognostic significance of stromal mast cells in canine mammary gland tumours

4.1 Introduction

An adequate number of canine mammary gland tumour (CMGT) cases with known disease outcome were identified from the surgical biopsy data base of IDEXX Laboratories, New Zealand, described in Chapter 3. The identified mammary tumour cases were then used to investigate the prognostic significance of tumour-associated inflammation related markers in CMGTs. These prognostic markers were adopted from previous human breast cancer studies and represented three categories: tumour infiltrating immune cells, chemokines and chemokine receptors and immune checkpoint molecules. This chapter describes the prognostic significance of tumour infiltrating immune cells in CMGTs. Of the different types of tumour infiltrating immune cells shown to influence the behaviour of human breast cancers, mast cells were selected for this study considering the abundance of them in CMGTs and simplicity of the techniques required to identify them.

Mast cells are frequently present in different types of human and animal cancers and many human cancer studies have investigated the prognostic potential of tumour associated mast cells.[3,9,37] These studies have shown that the presence of mast cells within a tumour influences tumour behaviour differently in different cancer types. For example, in gastric, pancreatic and colorectal cancers in people, a high intra-tumoural mast cell density correlates with a poor prognosis.[37] Conversely, oral squamous cell carcinomas and ovarian carcinomas with a high mast cell density have a more favourable prognosis.[4,37] Furthermore, mast cells in renal carcinoma and pulmonary small cell carcinoma in humans do not appear to influence the behaviour of the neoplasms.[37] Considering this seemingly variable role of mast cells in different types of human cancers, the ability of mast cell density to predict prognosis cannot be generalised and appears to be specific for each cancer type.[37] The variable influence of mast cells on tumour behaviour has been

suggested to be due to the high variety of chemical mediators produced by these cells, which have both pro- and anti-tumourigenic properties.[9,20]

The prognostic significance of mast cells in human breast cancer is currently not fully resolved[8,37], although most studies have revealed that high density of mast cells within a breast cancer is associated with a more favourable prognosis.[37] In dogs, four studies have investigated the presence of mast cells in CMGTs. One study showed significant differences in mast cell density between non-neoplastic mammary glands and neoplastic or dysplastic mammary glands.[34] In the other three studies, a positive correlation was observed between the mast cell density and tumour micro vessel density.[38,21,15] While these previous studies suggest that mast cells could have a role in CMGT development and progression, the prognostic potential of the presence of mast cells in CMGTs has not been previously evaluated. Therefore, the aim of the study described in this chapter was to investigate the density of mast cells in peripheral and stromal compartments of benign and malignant CMGTs. As the clinical outcome of each case was known, the mast cell densities of the tumours could be correlated with clinical outcome and survival times of the dogs to determine whether mast cell density is prognostic.

4.2 Material and methods

4.2.1 Case selection and assessment of survival times

This study included a subset of CMGT cases submitted for histopathology to IDEXX diagnostic laboratory, New Zealand, between 2012 and 2015, of which the contact details of submitting veterinarians were complete and current. In all cases, tumour surgical excision had been performed with a curative intent. Details regarding the patient signalment including age and reproductive status were identified from the surgical biopsy archive database. A questionnaire was sent to the submitting veterinarians to obtain other information. The information requested in the questionnaire included previous history of mammary gland disease, other concurrent disease conditions, the number of mammary

neoplasms present, abscessation or ulceration on the tumour surface, pre-surgical clinical exam findings, type of surgical procedure performed, additional treatments that had been used, evidence of mammary tumour metastasis, diagnostics used to detect tumour metastasis, and the cause of death for dogs that died. In addition, the submitting veterinarians were asked to provide the post-surgical clinical records of the dogs for at least three years from the date of surgery or until the date of the dog's death or euthanasia. Cases were excluded if adjunct therapies including anti-inflammatory drugs, steroids, cytotoxic chemotherapy or tyrosine kinase inhibitors were used to alter the neoplasm behaviour. Dogs which received antibiotics or vitamin supplements were not excluded. If the tumour surface was reported to be ulcerated or contained abscesses, those cases were excluded to avoid inclusion of inflammatory carcinomas. Using the post-surgical clinical records provided by the submitting veterinarians, the disease-specific survival time for each case was calculated retrospectively from the date of tumour excision to the date of the dog's death or euthanasia due to clinically-diagnosed mammary tumour metastasis.

4.2.2 Histology and mast cell quantification

Three micrometre sections were prepared from formalin-fixed, paraffin embedded (FFPE) mammary tumour tissue and stained with haematoxylin and eosin (HE) or toluidine blue (0.1% toluidine blue solution in 30% ethanol). The HE-stained sections were examined to determine the histological sub-types and grades of the tumours, following the guidelines of Goldschmidt et al. (2011) and Peña et al. (2013) respectively.[13,24] Briefly, simple carcinomas were graded according to three criteria: percentage tubule formation, nuclear pleomorphism, and mitoses/ 10 high power fields. In heterogeneous carcinomas, tubular scoring was assessed in the most representative malignant area. In addition, in complex and mixed tumours, the percentage of tubular formation was scored considering only epithelial areas with nuclear pleomorphism evaluated in all malignant components.

Tumours were classified into three groups: malignant-metastatic, malignant non-metastatic, and benign tumours. A tumour was classified as malignant-metastatic if the neoplasm was classified as malignant using histological criteria and had a clinical diagnosis of mammary tumour metastasis based on the development of radiographic lesions of pulmonary metastasis with other suggestive clinical signs of tumour metastasis during the follow-up period. Tumours were classified as malignant non-metastatic if the neoplasm was classified as malignant using histological criteria but had no clinical or radiographic evidence of metastases developed during the follow-up period. A tumour was classified as benign when the histology was consistent with a benign neoplasm. In addition to these three groups, a separate group of non-neoplastic mammary tissues was also included in the analysis. This group was comprised of non-neoplastic mammary gland tissue that had been submitted together with mammary gland neoplasms of some dogs.

Mast cell quantification was carried out using toluidine blue-stained sections, using a modification of a previously described method to evaluate mast cell density in human tissues.[31] Briefly, tumour peripheral and stromal areas with the highest mast cell density were identified by scanning the sections at low power (×100 magnification). Tumour periphery was defined as the area at the periphery of the tumour capsule in encapsulated tumours or the area immediately adjacent to the tumour margins in non-encapsulated tumours. Tumour stroma was defined as the inter- and intra-lobular and inter-ductal regions within the tumour. Individual mast cells were then counted in 10 non-overlapping high-power fields at ×400 magnification, where each microscopical field corresponded to an area of 0.785 $mm.^2$ This procedure was repeated twice for each tumour and then the average of the two counts was taken as the mast cell density of a mammary tumour. When non-neoplastic mammary gland tissues were available in addition to the neoplastic mammary gland, peripheral and stromal mast cell densities were also assessed in the non-neoplastic mammary gland tissue following the same method described above. When multiple wax blocks were available for a single large neoplasm, mast cells were counted in all sections and the average was taken. For the cases with multiple mammary neoplasms, the malignant tumour was used to assess the mast cell density.

4.2.3 Statistical analysis

Peripheral and stromal mast cell densities of the malignant–metastatic, malignant non-metastatic, benign tumours and non-neoplastic mammary tissues were compared using the Kruskal–Wallis H test to identify whether there were any significant differences between groups. The same test was used to identify the differences in stromal or peripheral mast cell densities in different mammary tumour histological sub-types and tumour grades. When significant differences were identified, post hoc analysis was performed by mean rank test to identify which group or groups were significantly different from the others. The association between stromal or peripheral mast cell density and survival time were analysed using Spearman's rank-order correlation. Other group comparisons were performed using Chi-squared or Fisher's exact tests. Survival times were investigated by Kaplan–Meier curves, and significant differences were determined by Log-rank test. These statistical analyses were performed using SPSS software Version 25 (IBM Corporation, Armonk, New York) and p values < 0.05 were considered to indicate significant differences. X-tile software (https://medicine.yale.edu/lab/rimm/research/software.aspx) was used to identify the optimal cut-off point for the stromal mast cell density that would best predict prognosis in dogs with malignant CMGTs.[5] A hierarchal multivariate analysis was performed to identify whether any tumour-related variables or stromal mast cell density were independently prognostic of the survival times of dogs with malignant mammary gland tumours. The tumour-related variables: tumour size, tumour histological grade, and the presence of intra-vascular or intra-lymphatic tumour emboli were included in the multivariate analysis because of their significant association with the survival times of the dogs with malignant mammary neoplasms in previous studies.

4.3 Results

4.3.1. Selected cases

From the 674 CMGT cases sourced from the surgical biopsy archive of IDEXX Laboratories, New Zealand, contact details of the submitting veterinarians were complete and current in 521 cases. Of them, the submitting veterinarians responded to the questionnaire in 201 cases while additional information regarding pre-surgical clinical exams and post-surgical follow-ups were available only in 63 cases. Ten of these 63 cases were excluded; the cause of death was not known in five dogs, there was insufficient follow-up time in three dogs, and two dogs had received anti-inflammatory drugs. Therefore, a total of 53 cases were available for prognostic analyses. The 53 CMGTs included 7 cases from 2012, 13 from 2013, 21 from 2014, and 12 cases from 2015. Of the selected 53 dogs, 36 (67.9%) were 5 – 10 years old, 14 (26.4%) were older than 10 years and age was unknown in three (5.5%) dogs. Forty-three (81.1%) dogs were intact female dogs while 10 (18.9%) dogs were spayed females. Of the selected 53 dogs, 51 (96.2%) dogs had solitary mammary neoplasms and two dogs had multiple neoplasms. Ten dogs had been diagnosed with other concurrent disease conditions at the time of diagnosis of mammary neoplasia including lipoma in two dogs and single cases of alopecia, hindlimb weakness, heart failure, dental diseases, ocular cyst, intervertebral disc disease, ceruminal cyst and debility due to old age. None of the dogs had any history of previous mammary gland disease, except one dog which was reported to have had a benign neoplasm that had been previously surgically removed. Simple mastectomies had been performed in 51 (96.2%) dogs while regional mastectomies had been performed in two dogs (3.8%).

4.3.2 Tumour size, histological sub-type, and grade

There were 10 (18.9%) small tumours (< 3 cm), 31 (58.5%) medium-sized tumours (3—5 cm) and 12 (22.6%) large tumours (> 10 cm). Forty one (77.3%) CMGTs were histologically classified as malignant while 12(22.7%) were benign tumours. The two dogs which had multiple neoplasms had two neoplasms in each: one histologically-benign neoplasm and

one histologically-malignant neoplasm. The malignant mammary tumours were classified into eight different histological sub-types with simple carcinomas further sub-classified into tubular, tubulopapillary, cribriform, and cystic papillary carcinomas (Table 4.1). Grading of the malignant tumours revealed that 13 were Grade I, 23 were Grade II and 5 were Grade III. Of the 53 neoplasms, 21 subsequently metastasised (therefore were classified as malignant-metastatic), 20 were malignant with no clinical evidence of metastasis (malignant non-metastatic), and 12 were benign. Intra-vascular or intra-lymphatic tumour emboli were observed in the HE-stained histological sections of five malignant mammary gland tumours all of which were in the malignant-metastatic group. An inflammatory cell infiltrate, predominantly present in the stromal compartment, was observed in all except three mammary gland tumours.

Table 4.1 Histological classification of canine mammary gland tumours.

Histological sub-type	
Malignant tumours	
Simple carcinoma	10
Tubular	5
Tubulopapillary	3
Cribriform	1
Cystic papillary	1
Intra-ductal papillary carcinoma	7
Adenosquamous carcinoma	6
Ductal carcinoma	6
Carcinoma-mixed	4
Carcinoma-complex	3
Solid carcinoma	2
Anaplastic carcinoma	2
Comedo carcinoma	1
Benign tumours	
Complex adenoma	5
Simple adenoma	3
Intra-ductal papillary adenoma	2
Ductal adenoma	1
Papillary adenoma	1
	53

4.3.3 Mast cell distribution

In most tumours mast cells were scattered along the pericapsular area and throughout the tumour stroma. Focal aggregates of mast cells were observed rarely in the neoplastic or non-neoplastic mammary tissues. In malignant CMGTs, mast cells were frequently found in the stromal and peripheral compartments of malignant non-metastatic tumours, while they were scarce in malignant–metastatic tumours (Figs. 4.1). There were no significant differences between stromal (p = 0.2) or peripheral (p = 0.47) mast cell densities between different mammary tumour histological sub-types (Kruskal-Wallis H test). Similarly, peripheral (p = 0.39) or stromal (p = 0.37) mast cell density did not differ between different mammary tumour grades (Kruskal-Wallis H test).

The lowest median stromal mast cell density (MCs/10HPFs) was observed in malignant–metastatic tumours (3 ± 37.4 (Quartile 3 – Quartile 1) followed by malignant non-metastatic tumours (69.6 ± 124.6 Q3-Q1), benign tumours (95 ± 71.5Q3-Q1) and non-neoplastic mammary tissues (107 ± 50 Q3-Q1; Table 4.2 and Fig. 4.1), with significant differences between groups (Z = 38.2, $p < 0.001$, Kruskal–Wallis H test). Post-hoc analysis revealed that mean rank of the stromal mast cell density in the malignant–metastatic CMGTs was significantly lower than the mean ranks of mast cell density in other groups (17.8 versus 51.8, 57.4 and 55.2, mean rank test). In contrast, there was no difference in peripheral mast cell density between the four groups (Z = 2.7, $p = 0.45$, Kruskal–Wallis H test)

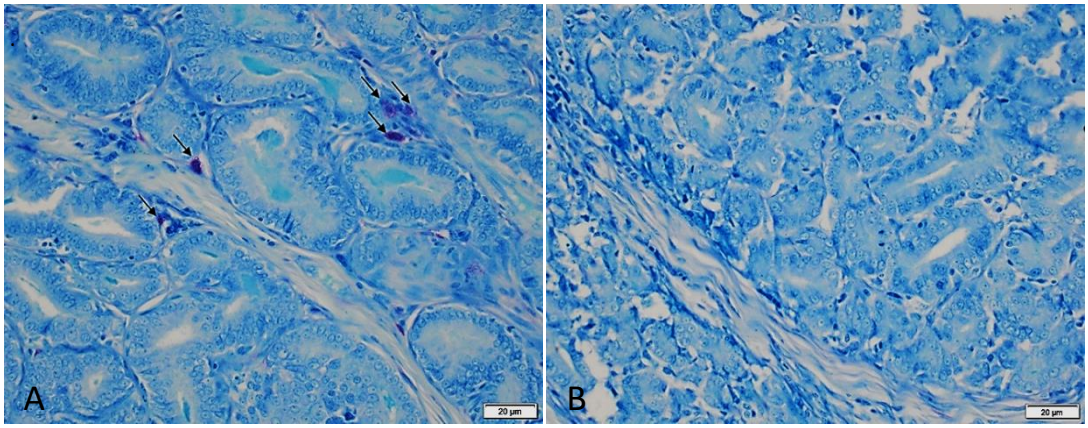

Figure 4.1 Grade II simple mammary carcinomas.

A: Note the abundant mast cells visible within the stroma (arrows) in this tumour, which did not develop subsequent metastases during the follow-up period. B: A grade II simple carcinoma which metastasised during the follow-up period. Note the lack of mast cells within the stroma of this tumour, Toluidine blue stain.

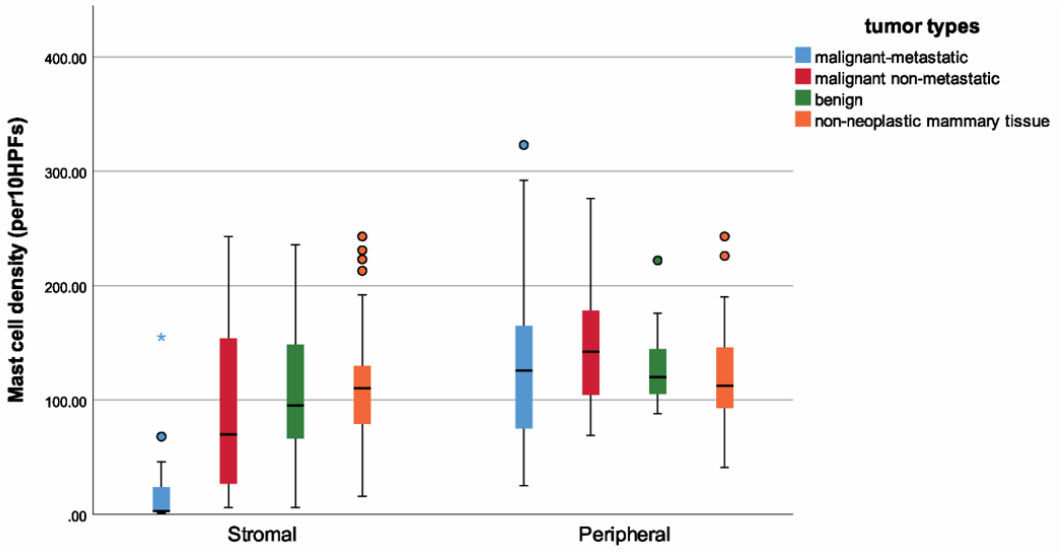

Figure 4.2 Clustered Boxplot of stromal and peripheral mast cell densities in different canine mammary gland tumour categories.

The box represents the first to third quartiles with the median indicated by the horizontal line. The vertical lines indicate the minimum and maximum values. Outliers are indicated by a circle, or an asterisk for extreme outliers greater than three times the interquartile range from first or third quartile.

Table 4.2 Peripheral and stromal MC densities in various categories of mammary gland tumours and non-neoplastic mammary tissue.

**Q3 – Q1 = Quartile 3 – Quartile 1*

	Number	Median ± (Q3-Q1*)	Z	*p* value
Peripheral mast cell density				
Malignant metastatic	21	126 ± 88.2		
Malignant non-metastatic	20	142 ± 72.2	2.7	0.45
Benign	12	95 ± 71.5		
Non-neoplastic mammary tissue	44	113.4. ± 51.6		
Stromal mast cell density				
Malignant metastatic	21	3 ± 22		
Malignant non-metastatic	20	69.6 ± 124.6	38.2	< .001
Benign	12	95 ± 71.5		
Non-neoplastic mammary tissue	44	107 ± 50		

4.3.4 Cut-off Analysis

By X-tile analysis, a strong, direct, and continuous association between stromal mast cell density and survival times of dogs with malignant CMGTs was identified. Furthermore, an optimal cut-off was identified as 10 MCs/10 HPFs ($\chi 2 = 20.13$), which best predicted the disease outcome. Therefore, a low stromal mast cell density was defined as ≤ 10 MCs/10 HPFs while a high stromal mast cell density was defined as > 10 MCs/10 HPFs.

4.3.5 Risk of tumour metastasis

In this study, 21/53 (39.6%) of dogs with malignant mammary tumours died after developing evidence of CMGT metastasis. In two of the 21 dogs, tumour metastasis was confirmed by post-mortem examination. Histology from one of these dogs revealed variably sized clusters of anaplastic epithelial cells arranged in glands within sections of lung, spleen, heart, and regional lymph nodes. Similar neoplastic cells were visible within sections of lung in the other dog in which necropsy examination was performed. In a further two of the 21 dogs in which metastases was diagnosed, the diagnosis was confirmed by cytology of fine needle aspirates of pulmonary masses identified in thoracic radiographs. Cytology from these cases revealed a population of anaplastic epithelial cells consistent with a malignant epithelial neoplasm. In the other 17 dogs in which metastasis was diagnosed, this diagnosis was made based on thoracic radiographical findings that revealed the presence of multiple solid masses in the pulmonary parenchyma accompanying an interstitial lung pattern. While these were not confirmed to be metastases microscopically, the radiographical findings in these cases were consistent with metastases and these neoplasms were classified as malignant–metastatic in this study. Moderate to severe regional lymphadenopathy was observed in clinical exams in 20 of the 21 dogs that had radiographic evidence of neoplasm metastasis including six dogs with enlarged lymph nodes that were reported to be fixed to the underlying tissues. However, lymphadenopathy was not further investigated using other diagnostics including cytological or histological examination due to the wishes of the owners. The percentage

deaths due to tumour metastasis 2- and 3-years after surgical excision of a malignant CMGTs were 41.6% (15/36) and 58% (21/36), respectively.

In 20 dogs which had histologically-malignant mammary neoplasms, radiographic evidence of tumour metastasis was not observed in pre- or post-surgical follow-ups. Except one dog which had mild popliteal lymph node enlargement which resolved in the next follow-up, post-surgical lymph node assessments were normal in all 19 dogs. Of these 20 dogs, four dogs died or were euthanised before the end of the three-year follow-up period due to unrelated causes. These four dogs died after 172, 423, 519 and 788 days of surgical excision of tumours. The causes included intervertebral disc disease in two dogs, age-related general debility in a single dog and one dog died following a road traffic accident. Another five dogs were lost for follow-up after three-years due to change in veterinary care provider while five dogs died or were euthanised due to unrelated causes after 4-6 years. The causes of death or euthanasia in these dogs included age-related general debility in three dogs, heart failure and degenerative joint disease in one dog each. The remaining six dogs were alive without any clinical records suggestive of tumour metastasis at the time of the retrospective survey was conducted for the present study.

The twelve dogs which had mammary neoplasms classified as histologically-benign were alive until the end of the three-year follow-up period. Three of them died after the three-year follow-up due to unrelated causes. Post-surgical lymph node assessments of all these 12 dogs were normal. Therefore, 21 dogs had mammary neoplasms which were classified as “malignant-metastatic”, 20 dogs had “malignant non-metastatic” neoplasms while 12 dogs had “benign” neoplasms. The median survival times of dogs classified to have malignant-metastatic neoplasms was 494 days while the median survival times of dogs with malignant non-metastatic and benign neoplasms were 1497 and 1717 days, respectively.

Rates of tumour metastasis were not significantly different between different histological sub-types (p = 0.13, Fisher’s exact test) or between different histological grades (p = 0.29,

Chi-squared test). According to the cut-off identified from X-tile analysis, 16 (43%) malignant mammary tumours had a low stromal mast cell density while 21 (57%) had a high stromal mast cell density. Thirteen of 16 (81%) dogs with malignant CMGTs with a low stromal mast cell density died of tumour metastasis during the follow-up period, while just 2 (9.5%) of the 21 dogs which had malignant CMGTs with high stromal mast cell density died due to tumour metastasis. Therefore, in this study, dogs with malignant CMGTs with low stromal mast cell density were approximately eight times more likely to die due to neoplasm metastasis than dogs with malignant CMGTs with a high stromal mast cell density.

4.3.6 Survival time analysis

The overall mean survival time (MST) of the 41 dogs with malignant CMGTs was 721 days (95% CI 609–833). There was a significant, moderate positive correlation between the stromal mast cell density and survival time (r_s = 0.50, p <0.001, Spearman's rank-order correlation), but no correlation between peripheral mast cell density and survival time (r_s = 0.13, p =0.41), Spearman's rank-order correlation). As part of the cut-off analysis performed using X-tile software, the survival times between dogs with malignant mammary tumours with low and high mast cell densities were compared. Dogs that had malignant mammary tumours with a low stromal mast cell density had a significantly shorter survival time (497 days 95% CI 342–651) than dogs with CMGTs that had a high stromal mast cell density (973 days, 95% CI 879–1,068, p < 0.001, X-tile analysis).

There were no significant differences between the MSTs of dogs with CMGTs of different histological sub-types (p = 0.08, Log-rank test, Table 4.3). However, there were significant differences in the MSTs of dogs with CMGTs of different grades (p = 0.001, Log-rank test). Post-hoc analysis showed that MST of dogs with grade I tumours (971 days, 95% CI 812–1,130) was significantly longer than the MSTs of dogs with grade II (729 days, 95% CI 585–872, p = 0.03) and grade III tumours (429 days, 95% CI 201–656, p < 0.001). Furthermore,

the MST of dogs with grade II tumours was significantly longer than that of dogs with grade III tumours (p = 0.04).

The hierarchical multivariate analysis showed that stromal mast cell density is prognostic of survival times of the dogs with malignant mammary neoplasms independent of tumour size, tumour grade and presence of intra-vascular or intra-lymphatic tumour emboli (ΔF = 8.4, p = 0.006). Tumour grade (ΔF = 6.3, p = 0.016) was also independently prognostic of survival times while neither tumour size ((ΔF = 1.2, p = 0.28) nor the presence of intra-lymphatic or intra-vascular tumour emboli (ΔF = 0.24, p = 0.68) were independently prognostic of survival times of dogs with malignant CMGTs.

Table 4.3 Survival times of dogs with mammary gland tumours.

	Number of dogs	Estimated Mean Survival Time (95% CI) Days	*p value*
Histological sub-type			
Total	29		
Simple carcinoma	10	809 (565-1053)	
Intra-ductal papillary carcinoma	7	1040 (943-1138)	0.08 (Log-rank test)
Adenosquamous carcinoma	6	610 (427-794)	
Ductal carcinoma	6	764 (457-1071)	
Histological grade			
Total	41		
Grade I	13	971 (812-1130)	
Grade II	23	729(585-872)	0.001 (Log-rank test)
Grade III	5	429(201-656)	
Mast cell density-Malignant tumours			
Stromal MCD			
≤ 10/10 HPFs	16	497 (342-651)	< 0.001 (X-tile analysis)
>10/10 HPFs	25	973 (879-1068)	

4.4 Discussion

In the present study, the behaviour of the CMGTs could be predicted by the density of the mast cells in the tumour stroma. Stromal mast cell density was significantly lower in malignant tumours where the patient later developed metastasis, compared with malignant tumours that did not subsequently metastasise during the follow-up period. Furthermore, there was a significant correlation between stromal mast cell density and the survival time of dogs with malignant mammary gland tumours while stromal mast cell density was independently prognostic of survival times of dogs with malignant neoplasms. In addition, dogs with malignant CMGTs classified as having low stromal mast cell density were eight times more likely to die due to tumour metastasis than dogs with malignant CMGTs with a high stromal mast cell density. These results therefore suggest that stromal mast cell density is an important prognostic indicator for CMGTs.

Similar to the results in the present study of CMGTs, mast cell density has been found to be prognostic in the majority of human breast cancer studies.[1,2,28,12,16] Interestingly, while most studies of human breast cancer have reported that a high stromal mast cell density was indicative of a favourable prognosis, a small number of studies have associated high stromal mast cell density with an unfavourable prognosis.[17,29,19] These apparently contradictory results suggest that stromal mast cells may influence tumour behaviour differently in some circumstances. Three studies have investigated whether stromal mast cell density in breast cancer is associated with other molecular prognostic factors including the presence of oestrogen or progesterone hormone receptors or human epidermal growth factor receptor–2.[2,12,31] These studies produced conflicting results and it is currently unclear whether or not stromal mast cell density is a prognostic factor that is independent of the other molecular factors currently recognised as prognostic for human breast cancer. There are currently no studies investigating an association of hormone or growth factor receptor expression and mast cell density in canine mammary gland tumours.

The mean overall survival time of the dogs with malignant CMGTs included in this study was 721 days. This is consistent with the MSTs reported by previous studies on these neoplasms which ranged between 359–720 days.[6,30,32] Previous studies conducted in countries including Sweden, Greece, Spain and Italy have reported that 41–80% of dogs with malignant CMGTs survive for at least 2 years after neoplasm excision.[14,18,24,33] Similarly, the 2-year survival rate in the present study was 59%. The similarities in survival times and survival rates between the present and previous studies of CMGTs suggest that the 41 malignant neoplasms included in the present study were representative of malignant CMGTs in the wider population of dogs. Therefore, although the present study contained comparatively small numbers of CMGTs, it is expected that stromal mast cell density will be also associated with CMGT behaviour in larger samples of these tumours.

In the present study, tumour histological grade was identified to be independently predictive of survival times of the dogs with malignant canine mammary neoplasms. In this study and in several previous studies, tumour grade was shown to be prognostic of disease outcome or survival times of dogs with malignant CMGTs in multivariate analyses.[18,24] Unlike tumour histological grade, tumour size or presence of tumour emboli were not independently predictive of the survival times of the dogs in the present study. Tumour size has been identified to be prognostic of CMGTs in previous studies by multivariate analysis.[30] The lack of significance in the present study could be due to small number of large-sized neoplasms. Although the presence of intra-lymphatic or intra-vascular tumour emboli is generally suggestive of more aggressive disease, detection of tumour emboli in histological sections is dependent on several factors such as number of histological sections prepared from a neoplasm. Therefore, the lack of significance identified in the present study could be a result of that. Alternatively, it is possible that presence of tumour emboli is prognostic of disease outcome of malignant CMGTs but not independent of other prognostic factors.

Survival times were not significantly different between different histological sub-types of mammary tumours in this study. However, there were only small numbers of some of the

less common sub-types included in this study. Therefore, histological sub-type was not included in the multivariate analysis. Further studies with sufficiently large numbers of tumours in each histological subtype are necessary to investigate the impact of histological sub-type on prognosis.

Currently, tumour grade and histological classification are recommended for prognostic determination in CMGTs.[23,35] However, the subjectivity of tumour classification and grading has been identified as a disadvantage due to high interobserver variability.[7,22] Therefore, histological classification and grading need to be complemented with other reliable histochemical or molecular methods to improve the prognostic accuracy for CMGTs. Advantages of assessing stromal mast cell density include the low cost of toluidine blue-staining compared to other immunohistochemical or molecular methods used to determine prognostic markers for CMGTs[25,27,32], the ease of recognising mast cells within the toluidine blue-stained sections, and the ability to objectively count the numbers of cells in histological fields. Due to the ease of identification of mast cells, using these cells to predict prognosis would appear to be readily adaptable to automated counting of the cells within a histological field. Additionally, the simplicity and low cost of toluidine blue staining method would facilitate the incorporation of measuring stromal mast cell density into the routine assessment of prognosis for CMGTs.

The present study revealed a continuous positive correlation between stromal MC density and survival times of dogs with malignant CMGTs. Despite the continuous nature of the association, it is practical to have cut-offs that can be communicated to clients in a commercial setting. In this study, ≤ 10/10 HPFs was identified as the optimal cut-off for stromal mast cell density with 81% of the dogs with malignant CMGTs with a stromal mast cell density ≤ 10/10 HPFs developing tumour metastasis, while metastasis developed in only 9.5% of the dogs with malignant CMGTs that had a mast cell density of > 10/10HPFs. Therefore, determining whether a malignant CMGT has more or less than 10 stromal MCs/10HPFs would be comparatively easy for pathologists, but appears to be a powerful

predictor of prognosis and therefore which dogs are most likely to benefit from post-surgical adjuvant therapy to prevent subsequent tumour metastasis.

The results of the present study revealed that stromal mast cell density predicted the biological behaviour of CMGTs. However, it is unknown whether the stromal MC density directly influences tumour metastasis or whether both the mast cell density and the behaviour of the neoplasm are determined by the properties of the neoplastic cells. If mast cells influence tumour behaviour, they could do it by producing anti-tumour compounds that prevent tumour metastasis.[36,37,10,3] For example, chondroitin sulphate secreted by MCs may increase adhesion between tumour cells and the extracellular matrix and therefore inhibit tumour metastasis.[10] In addition, heparan sulphate proteoglycans secreted by MCs inhibit neovascularisation in tumours, minimising the possibility of tumour metastasis.[36]

In the present study, peripheral MC density was not associated with disease outcome. It is not certain what mechanisms operate differently between the tumour stromal and peripheral compartments to produce this discrepancy. However, stromal mast cells are located within the tumour and therefore more closely associated with the tumour cells and tumour microenvironment than the MCs scattered along the tumour periphery. Typically, the disease outcome of a tumour is determined by the properties of the tumour cells and tumour microenvironment.[26,11] Therefore, stromal mast cells which are in close association with tumour cells and the tumour microenvironment are more likely to influence tumour behaviour and be prognostic of the disease outcome than peripheral mast cells.

Only two previous studies have investigated the mast cell density within normal or neoplastic canine mammary glands. In contrast to the present study, mast cell density of non-neoplastic mammary tissues in both previous studies was lower than that of neoplastic mammary gland.[34,15] The reasons for this difference is unclear, although both previous studies contained small numbers of samples and only determined the overall

mast cell density, rather than distinguishing between peripheral and stromal compartments.

In conclusion, these findings suggest that measuring stromal mast cell density using toluidine blue staining may represent an easy-to-perform and cost-effective histopathological parameter that, in conjunction with classification and grading, could better predict the behaviour of canine mammary neoplasms.

4.5 Bibliography

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Chapter 5 : Prognostic significance of chemokines and chemokine receptors in canine mammary gland tumours

5.1 Introduction

In the previous chapter, the potential of using stromal mast cell density for prognostic determination in canine mammary gland tumours (CMGTs) was investigated. Like tumour stromal mast cell density, many studies have investigated the potential of using chemokines and chemokine receptor expression in tumour cells for prognostic determination in human breast cancers.[7,30] Chemokines are small molecular weight signalling proteins secreted by immune cells that control migration and positioning of immune and inflammatory cells within the body.[10] Although the primary function of chemokines is leukocyte trafficking, recent research suggests they also influence growth, progression and metastasis of many human cancers.[30] Some human cancer cells have been shown to produce chemokines while other studies have identified abnormal expression of chemokine receptors by neoplastic cells.[20] Breast cancer cells have been shown to express chemokine receptors and increased expression of some chemokine receptors has been associated with a worse disease outcome.[20] Additionally, some breast cancer cells have also been reported to produce chemokines with increased expression of some chemokines associated with a more aggressive clinical course and a worse disease outcome.[33]

Many similarities have been identified between human breast cancers and canine mammary gland tumours including a hormonal influence on development, histopathologic features, expression patterns of some molecular markers, and an unpredictable clinical course.[14,27] As in humans, gene expression of chemokines and chemokine receptors has been evaluated in CMGTs with two studies finding higher expression of some chemokines and chemokine receptors in CMGTs that were histologically classified as malignant compared to CMGTs that were histologically classified as benign or normal mammary gland tissue adjacent to the mammary neoplasm.[2,6] However, whether or not the

expression of chemokine or chemokine receptors influence the biological behaviour of CMGTs has not been previously investigated. Therefore, the aim of this chapter was to evaluate gene expression of five chemokine receptors, CXCR3, CXCR4, CXCR7, CCR4, CCR9 and three chemokines CXCL10, CXCL12, CCL5 in a series of 41 malignant and 12 benign CMGTs identified from the retrospective survey described in Chapter 3. These chemokines and chemokine receptors were selected because they have been extensively studied in humans and have been shown to influence the biological behaviour of human breast cancers.[31,17,15,18,23,13,21,36] As the disease outcome of the CMGTs included was known, chemokines and chemokine receptor gene expression could be compared in neoplasms that subsequently metastasised with those that did not. Additionally, it could be determined if chemokine and chemokine receptor expression was associated with survival times of these dogs. The identification of an association between chemokine and chemokine receptor gene expression and disease outcome would suggest that chemokine and chemokine receptor gene expression may, as in human breast cancers, influence tumour behaviour.

5.2 Material and methods

5.2.1 Case selection and assessment of survival times

The same set of canine mammary tumour cases described in Chapter 4 was used for this study. Briefly, mammary gland tumour cases submitted to IDEXX diagnostic laboratory, New Zealand, for histopathology between 2012 and 2015 were sourced from the laboratory surgical biopsy archive. Details regarding the patient signalment were identified from the IDEXX surgical biopsy archive while the clinical records of the patients and information regarding the post-surgical follow-ups were obtained by contacting the submitting veterinarians. Cases were excluded if adjunct therapies including anti-inflammatory drugs or steroids were used to alter the neoplasm behaviour or if the tumour surface was reported to be ulcerated or contained abscesses. The disease-specific survival time for each case was calculated retrospectively from the date of tumour excision to the date of the dog's death or euthanasia due to clinically-diagnosed mammary tumour metastasis.

5.2.2 Histological classification and grading

The histological classification and grading of the canine mammary neoplasms was performed as described in section 4.2.2 in Chapter 4. Tumours were classified into three groups; malignant-metastatic, malignant non-metastatic and benign following the criteria described in the same section.

5.2.2 RNA extraction

For RNA extraction, three 10 µm tissue sections were cut from each formalin-fixed paraffin-embedded (FFPE) mammary tumour tissue and placed on glass slides. In the tissue sections which contained moderate amounts of intervening stroma, the sections were placed on glass slides but left unstained and unfixed. They were then viewed under a dissecting microscope and neoplastic cells were carefully scraped off using a clean scalpel blade into 1.5 mL microtubes for RNA extraction, using the HE stained section of the same specimen as a guide. This step was used to ensure that all tissue sections used for RNA extraction contained at least 80% tumour tissue. When neoplastic cells of various origins were present on a single section, all neoplastic cells were included for RNA extraction without discrimination. Before RNA extraction, all the equipment used, as well as the surface of the laboratory bench, was cleaned with RNAse decontamination solution (RNAseZap, Sigma-Aldrich, MO, USA). Total RNA was extracted from the samples using the Nucleospin totalRNA FFPE XS kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and nucleic acid concentrations were quantified using a Qubit 2.0 fluorometer and assay kit (Life Technologies, Carlsbad, CA, USA). For the cases which had more than one FFPE block for a single tumour, RNA extraction was performed from each block separately and the extracts were mixed prior to further assessments. For cases that had more than one mammary neoplasm, tissue block from the malignant tumour was used for RNA extraction. To remove any residual DNA, post-extraction DNAse digestion was performed using Ambion Turbo DNA-free DNAse following the manufacturer's instructions (Life Technologies). Complementary DNA synthesis was carried out with the

Transcriptor first strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) using 0.5 µg total RNA, and both random hexamer and oligo-dT primers.

5.2.3 RT-PCR

Five chemokine receptors including CXCR3, CXCR4, CXCR7, CCR4, CCR9 and three chemokines CXCL10, CXCL12, and CCL5 were selected for gene expression analysis. To normalize the gene expression between CMGT samples of varying quantity and quality, HPRT and RPL32 reference genes were used. For all the selected genes except CXCR7, previously published primer sequences were used (Table 5.1).[4,16,22,11]

For CXCR7, new primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and mfold (http://unafold.rna.albany.edu/?q=mfold) and the newly developed assay was validated. To validate the specificity of the primers, positive control cDNA samples were prepared using RNA extracted from a lymph node of a dog that had died of an unrelated cause. The product amplified with the new CXCR7 primers was purified and sequenced. The sensitivity of the new CXCR7 assay was determined using three ten-fold serial dilution assays of the amplicon while the linearity of the assays was determined from the efficiency and r^2 values calculated using the curves generated from the dilution assays. Precision was evaluated by calculating intra-assay variability based on the distribution of linearised Ct values for five replicates of each standard in a single PCR run. Reproducibility was evaluated by calculating inter-assay variability comparing the linearised Ct values obtained for the same standards in the three separate dilution assays. The intra- and inter-assay variability was expressed as a coefficient of variance (CV), which was the ratio of the standard deviation to the mean of the linearised Ct values for a particular standard, expressed as a percentage.

All real-time PCR assays (RT-PCR) were performed using a Mic qPCR Cycler (Bio Molecular System, Upper Coomera, Australia). The RT- PCR reactions were performed using AccuMelt HRM SuperMix (Quanta Biosciences, Gaithersburg, MD), using 10 ng of cDNA with 0.5 µM forward and reverse primer concentration in a total volume of 10 µL reaction mix. All reactions were performed in duplicate and each plate included a positive control and a no

template control. Residual genomic DNA was excluded on the basis of the melting temperature and/or minus-RT controls. Reference gene stability was analysed using GeNorm software.

Table 5.1 Primer sequences of the chemokines and chemokine receptors used in this study

Gene	Gene bank Accession No:	Primers (5' - 3')	bps	Tm (°C)	Reference
CCL5	NM_001003010.2	F: AAGGGCTGACTGATAAATGTGA R: AGCGAGAATTTTAATGGAAAGC	52	51	*Nascimento et al., 2013*
CXCL10	AB183191.1	F: CACATGTTGAGATCATTGCCA R: TTCAGACATCTTTTCTCCCCA	62	54	*Nascimento et al., 2013*
CXCR4	NM_001048026.1	F: GAGCGGTTACCATGGAAGAG R: CGGTTGAAGTGAGCATTTTCC	108	54	*Im et al., 2017*
CXCR7	NM_001003281.2	F: TTGGAGCAAAACGCCAAGTG R: TCTTGGAGACGATGCAACCC	92	56	Designed primers
CXCL12	NM_001128097.1	F: TCTTCGAGAGCCACAT TGC R: TTCAGTCTTGCCACGAT CTG	82	57	*Im et al., 2017*
CCR9	XM_541909	F: CACTTCCTCCCACCCTTGTA R: TGGTCTTGACTCTGGTGCAG	100	56	*Maeda et al., 2011*
CXCR3	AB185149.1	F: TTCTTTGCCATCCCAGATTTC R: ATGCATGGCATTTAGGCG	67	53	*Nascimento et al., 2013*
CCR4	NM_001003020.1	F: TTTGGACTAGGTCTCTGCAAGA R: AAAAGCCCACCAGGTACATC	52	55	*Nascimento et al., 2013*
RPL32	XM_848016.1	F: TGGTTACAGGAGCAACAAGAA R: GCACATCAGCAGCACTTCA	100	54	*Maeda et al., 2011*
HPRT	AY283372	F: AGCTTGCTGGTGAAAAGGAC	114	56	*Brinkhof et al., 2006*

5.2.4 Relative quantification of the chemokine and chemokine receptor gene expression in mammary tumour samples

For each gene, the melting temperatures and the shapes of the melting curves of the samples were compared with the corresponding positive controls. The samples which had melting temperatures within the range of ± 1.5 °C of the melting temperature of the positive controls were selected for the analyses. When multiple runs were included for a single gene, the CV of the cycle threshold (Cts) of the positive controls were calculated and only considered appropriate for analysis if the CVs were < 20%.

Relative expression of genes of interest in each CMGT was analysed using ΔΔCt method. Briefly, for each sample, the difference (ΔCt) between the Ct value of the gene of interest and the average Ct value of the two reference genes were calculated. Then a ΔΔCt value was calculated by taking the difference between the calculated ΔCt value of each sample and the average ΔCt value of the control group, considering the non-metastatic malignant mammary gland tumours as the control group.

5.2.5 Statistical analysis

The correlation between the relative expression of genes of interest and the survival times of the dogs was analysed by Spearman rank-order correlation test. Kruskal-Wallis H test was used to compare the gene expression between malignant-metastatic and malignant non-metastatic CMGTs, and different histological grades of CMGTs. Samples with detectable reference gene expression but without detectable expression of the gene of interest were considered as negative for the particular gene. When a gene had a high number of negative samples, gene expression data was converted to a binary positive or negative result. Pearson Chi-Squared test was used for the group comparisons in these genes instead of Kruskal-Wallis H test as the former allowed inclusion of negative samples into the statistical analysis. The differences in survival times between gene expression positive and gene expression negative groups were compared using Kaplan-Maier survival

curves and Log-rank test. A hierarchal multivariate analysis was performed to identify which tumour-related variables and chemokine, or chemokine receptor genes independently predicted the survival times of dogs with malignant mammary gland tumours. The tumour-related variables tested in multivariate analysis included tumour size, tumour histological grade, and the presence of intra-vascular or intra-lymphatic tumour emboli. The chemokines or chemokine receptors included in the multivariate analysis were selected considering the significant correlation between their expression and survival times of dogs identified in the present study. All statistical analyses were performed using the SPSS version 25 program (IBM Corporation, Armonk, NY, USA). *P* values less than < 0.05 were considered to be indicative of statistically significant differences.

5.3 Results

5.3.1 Selected cases

The clinicopathological characteristics of patients are described in section 4.3.1 of Chapter 4.

5.3.2 Tumour size, histological sub-types and grades

The characteristics of the tumours are described in 4.3.2 of Chapter 4.

5.3.3 Risk of tumour metastasis

The details of mammary tumour metastasis in the dogs included in the study are described in section 4.3.5 of Chapter 4. Briefly, 21/41 (51.2 %) dogs with malignant mammary tumours died after developing evidence of tumour metastasis within three years of surgical excision of the neoplasms. The other dogs 20 (48.8%) with malignant neoplasms were not diagnosed to have clinically evident mammary tumour metastasis during the 3-year follow-up period. The patient and tumour characteristics of the cases included in

malignant-metastatic, malignant non-metastatic and benign groups are summarised in Table 5.2.

Table 5.2 Clinical characteristics of the patients and gross and histological characteristics of the neoplasms included in the malignant-metastatic, malignant non-metastatic and benign canine mammary gland tumour categories

	Malignant-metastatic	Malignant non-metastatic	Benign
Number of cases	21	20	12
Age			
5 - 10 yrs	15	10	11
> 10 yrs	6	7	1
Unknown	0	3	0
Reproductive status			
Intact	16	16	11
Spayed	5	4	1
Number of tumours			
Single	19	20	12
Multiple	2	0	0
Tumour size			
Small (<3cm)	0	0	10
Medium (3-5cm)	11	18	2
Large (>5cm)	10	2	0
Histological type			
	Simple carcinoma (5) Adenosquamous carcinoma (5) Ductal carcinoma (3) Carcinoma - Mixed type (3) Carcinoma - solid (2) Comedo carcinoma (1) Carcinoma - Anaplastic (1) Intra-ductal papillary carcinoma (1)	Intra-ductal papillary carcinoma (6) Simple carcinoma (6) Complex carcinoma (3) Ductal carcinoma (3) Adenosquamous carcinoma (1) Carcinoma - Anaplastic (1)	Complex adenoma (7) Simple adenoma (3) Papillary adenoma (2)
Histological grade			
Grade I	3	10	N/A
Grade II	14	9	N/A
Grade III	4	1	N/A
Presence of tumour emboli	5	0	0

5.3.4 Assay validation of CXCR7 and reference gene stability

The newly developed CXCR7 gene expression assay was linear within the tested range from 10^2 to 10^7 target copies with an efficiency of 1.035 and r^2 value of 99.85%. The CV of the mean linearized Ct values obtained with five replicates of different dilutions of the standard in a single test run ranged from 10.1—17.9%. The CV of the mean linearized Ct values obtained in three separate runs of the test ranged from 7.4—19.5%. This indicates adequate precision and reproducibility of the assay. The average geNorm M value was ≤ 0.2 indicating a high reference gene stability. The geNorm V value was < 0.15 suggesting that the pair-wise variation between the two reference genes HPRT and RPL32 is minimal and these two reference genes can be reliably used to normalise the gene expression between samples.

5.3.5 Relative gene expression of chemokines CCL5, CXCL12 and CXCL10 in CMGTs

In the 41 malignant CMGTs included in the study, reference gene expression was positive in 40 tumours and undetectable in one. This mammary tumour was excluded from further analyses. Analysis of the relative quantities of gene expression was only performed for genes in which a high proportion of the tumours had detectable expression of the gene of interest. This included three chemokines: CCL5, CXCL10 and CXCL12. For CXCL12 target gene expression was observed in 39 (97%) CMGTs. One sample was excluded due to absence of CXCL12 expression. CCL5 expression was observed in 38 (97.5%) mammary tumours. One tumour did not have detectable expression and the other was excluded due to inappropriate melting peak which suggests non-target amplification. Detectable CXCL10 expression was observed in 26 (66.7%) mammary tumours and undetectable in six. Eight tumours were excluded from CXCL10 gene expression analysis due to other reasons; the melting temperatures were not appropriate in six tumours, and in two tumours only one replicate was positive.

For CCL5, the ΔΔCt values in malignant CMGTs ranged from -3.97 to 7.08. The mean ranks of ΔΔCt of CCL5 in malignant-metastatic tumours was significantly different from that of malignant non-metastatic tumours (Kruskal-wallis H test, $Z = 4.3$, $p = 0.038$). For CXCL12, the ΔΔCts in malignant CMGTs ranged from -3.61 to 1.13 and the mean ranks of ΔΔCt values between metastatic and non-metastatic mammary tumour groups was significantly different (Kruskal-wallis H test, $Z = 12.4$, $p < 0.005$). In contrast, expression of CXCL10 was not different between the two groups ($p = 0.76$). There were no significant differences in relative gene expression of CCL5, CXCL12 and CXCL10 genes between different histological grades of CMGTs. Gene expression between different histological sub-types was not evaluated due to small number of samples included in some categories.

5.3.6 Gene expression of chemokine receptors: CXCR3, CXCR4, CXCR7, CCR4 and CCR9 in CMGTs

Gene expression data for chemokine receptors was considered positive when target gene expression was detected in the tumour, or negative when target gene expression was not detected in the tumour despite adequate expression of the reference genes. Although CXCR3, CXCR4, CXCR7 and CCR4 genes had an adequate number of mammary tumours with detectable target gene expression, the data could not be reliably quantified as the Ct values of some of the samples were not within the linear range of the assay. For CCR9 gene, the number of positive tumours was low. Therefore, relative gene quantification was not performed for these genes and tumours were considered on positive or negative basis for statistical analysis.

Chemokine receptor CXC 3 (CXCR3) gene expression was detected in 23/39 malignant CMGTs, with no CXCR3 expression detected in 16 tumours. One tumour was excluded due to an inappropriate melting peak. Of the 20 CMGTs that subsequently metastasised, 15 (75%) were positive for CXCR3 expression while CXCR3 expression was detected only in 8/19 (42%) of the CMGTs which did not develop metastases (Table 5.3). Therefore, CMGTs

with positive CXCR3 expression metastasised significantly more frequently in this study than CMGTs without CXCR3 expression (Chi-squared test, $p = 0.037$). Positive CXCR4 expression was identified in 20/38 malignant CMGTs samples and 18 samples were negative for CXCR4. Two samples were excluded due to inappropriate melting peaks. Fourteen (70%) metastatic tumours had positive expression of CXCR4 while only 6/18 (33%) non-metastatic samples showed positive expression of CXCR4. Therefore, a significantly higher proportion of metastatic malignant CMGTs were positive for CXCR4 expression compared to the non-metastatic malignant CMGTs (Chi-squared test, $p = 0.026$). Positive CXCR7 expression was observed in 19/38 mammary tumours while it was negative in 19 tumours. Two samples were excluded due to inappropriate melting peaks. The proportion of metastatic malignant CMGTs which had positive CXCR7 expression (13/19, 68%) was significantly higher than the proportion of CXCR7 positive malignant tumours which did not subsequently metastasise (6/19, 32%) (Chi-squared test, $p = 0.025$). Only 33 CMGTs could be included in the CCR9 assay. Six samples had insufficient amplifiable RNA and therefore could not be assayed. One sample was excluded due to an inappropriate melting peak. Of the 14 metastatic mammary tumours included, 8 (57%) tumours had positive CCR9 expression while only 4/19 (21%) non-metastatic CMGTs had positive CCR9 expression. Therefore, a significantly higher proportion of CMGTs which had developed tumour metastases had positive CCR9 expression ($p = 0.039$) compared to those that did not develop metastasis during the follow-up period. Twenty samples were positive for CCR4 expression while 17 samples were negative. Four samples were excluded due to inappropriate melting temperatures. In contrast to the other chemokines and chemokine receptors, there was no significant difference between the proportions of CCR4 positive tumours in the metastatic (7/16, 45%) and non-metastatic (13/20, 65%) CMGT groups (Chi-squared test, $p = 0.17$).

Table 5.3 Gene expression analysis for chemokine receptors.

**For each gene, 1–5 samples were excluded for inappropriate melting peaks. Six mammary tumours could not be included in CCR9 assay due to insufficient sample volumes.*

		Proportion of malignant CMGTs with positive target gene expression		
Chemokine receptor	Total*	Metastatic	Non-metastatic	Chi-sq *p* value
CXCR3	23/39 (56%)	15/20 (75%)	8/19 (42%)	0.037
CXCR4	20/38 (55%)	14/20 (70%)	6/18 (33%)	0.026
CXCR7	19/38 (50%)	13/19 (68%)	6/19 (32%)	0.025
CCR9	12/33 (36%)	8/14 (57%)	4/19 (21%)	0.039
CCR4	20/36 (55%)	7/16 (45%)	13/20 (65%)	0.17

5.3.7 Gene expression and survival times of dogs

Analysis of the correlation between chemokine gene expression (relative quantity) and survival time in the dogs with malignant CMGTs found a statistically significant, moderate negative correlation between CXCL12 gene expression and survival times (Spearman's rank-order correlation r_s = -0.40, p = 0.03). There was also a moderate, negative correlation between CCL5 expression and survival times of dogs (Spearman's rank-order correlation, r_s = -0.40, p = 0.02). However, there was no significant correlation between CXCL10 expression in CMGTs and survival time (Spearman's rank-order correlation, r_s = 0.27, p =0.38).

The overall mean survival time (MST) of the 41 dogs with malignant CMGTs was 721 days: (95% CI 609 – 833). When dogs were grouped according to chemokine receptor gene expression, the mean survival time differed significantly between groups of some chemokine receptors but not others. The mean survival time (MST) of the dogs with CXCR4 positive tumours was significantly lower (623 days, 95% CI 455—793) than that of the dogs with CXCR4 negative mammary gland tumours (Log-rank test, 845 days, 95% CI 688-1002, p = 0.045, Figure 5.1). Similarly, the MST of the dogs with CCR9 positive tumours was 686 days (95% CI 488-885) which was significantly lower than the MST of CCR9 negative dogs (Log-rank test, 817 days: 95% CI 750—1039, p = 0.039). In contrast, the differences of

MSTs between the chemokine receptor positive and negative groups were not significant for CXCR3, CXCR7 and CCR4 chemokine receptors.

A hierarchal multivariate analysis was performed which included the following chemokines and chemokine receptors: CCL5, CXCL12, CXCR4 and CCR9. These chemokines and chemokine receptors were selected for analysis because their expression was significantly associated with survival times of the dogs, through either relative quantification (CCL5 and CXCL12) or simply the presence or absence of gene expression (CXCR4 and CCR9). In addition to the chemokines and chemokine receptors, tumour size, tumour grade and presence of tumour emboli in histological section were also included as independent variables. Of these variables, histological grade (ΔF= 4.3, p = 0.048) and CCL5 gene expression (ΔF= 5.7, p = 0.026) were identified as independently predicting the survival times of the dogs with malignant mammary neoplasms (Table 5.4).

Table 5.4 Hierarchical multivariate analysis.

*The variability of the survival times predicted by each variable is denoted by ΔF and p values indicate the significance of ΔF. **p < 0.05*

Independent variables	*Δ F*	*p*
Tumour size	0.365	0.551
Tumour histological grade	4.301	0.048**
Presence of tumour emboli	2.528	0.124
CCL5	5.665	0.026**
CXCL12	0.522	0.477
CXCR4	1.293	0.168
CCR9	0.251	0.89

**$p < 0.05$

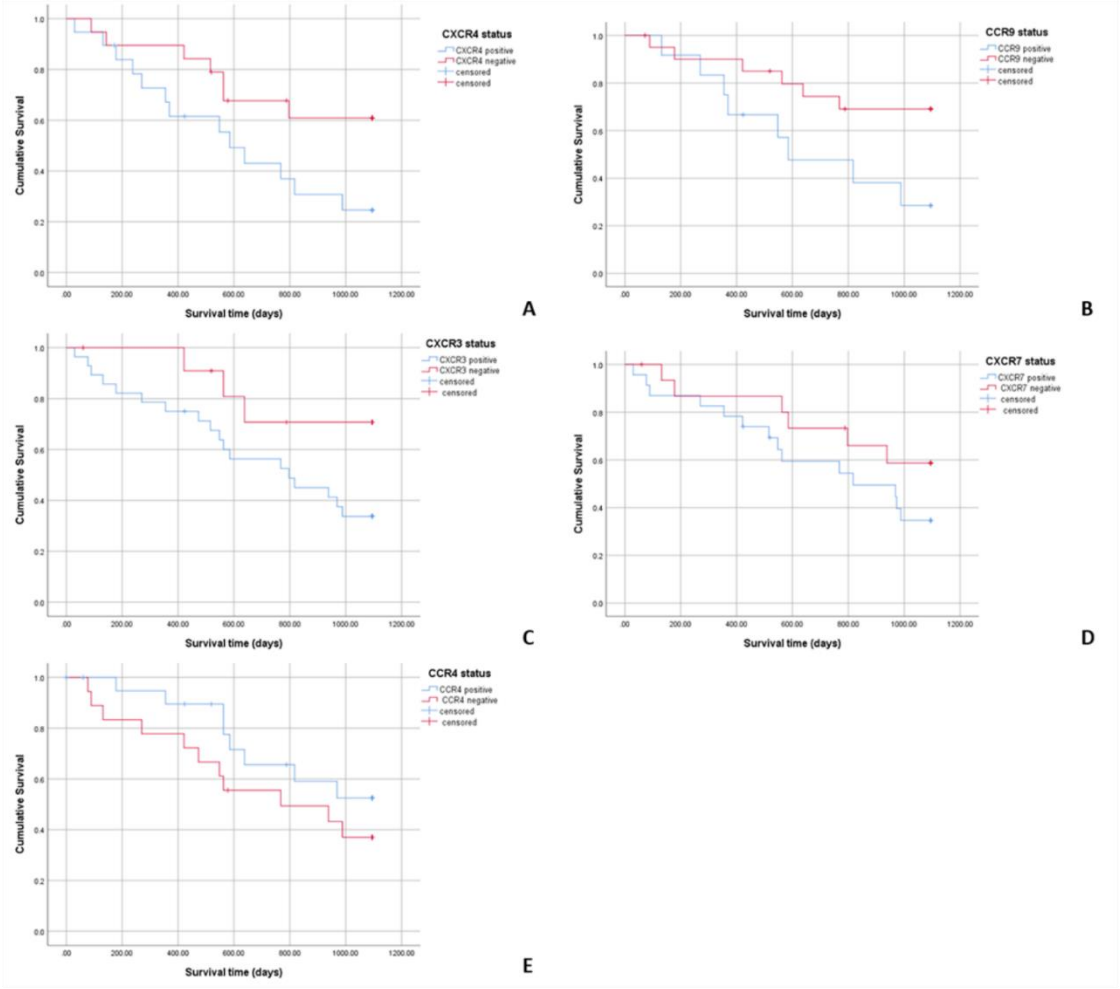

Figure 5.1 Kaplan-Meier survival curves of the dogs.

A: Dogs with mammary gland tumours positive and negative for CXCR4. The mean survival times between dogs which had malignant mammary tumours which were positive for CXCR4 was significantly different from that of tumours which were negative for CXCR4 ($p = 0.039$) B: Dogs with mammary gland tumours positive and negative for CCR9. The mean survival times between dogs which had malignant mammary tumours which were positive for CCR9 was significantly different from that of tumours which were negative for CCR9 ($p = 0.018$). Dogs with mammary gland tumours positive and negative for CXCR3 (C), CXCR7 (D) and CCR4 (E). For C, D and E the mean survival times of dogs with positive and negative tumours were not significantly different ($p > 0.05$).

5.3.8 Gene expression in benign CMGTs

In regard to chemokine gene expression in the 12 benign CMGTs, expression of CCL5 was identified in 6/12 (50%) mammary tumours, CXCL10 expression in 7 (64%), and CXCL12 expression in only one benign neoplasm (10%). However, even when chemokine gene expression was detected in benign tumours, this expression was very low and outside of the linear ranges of the assays. This is in contrast with the malignant CMGTs where chemokine gene expression was present in the majority of samples and was higher, being within the linear range of the assays. Regarding chemokine receptor gene expression (CXCR3, CXCR4, CXCR7, CCR4 and CCR9) only two benign tumours from each gene had detectable gene expression. Further, for all the chemokine receptor genes Chi-squared analysis showed that the proportion of benign CMGTs with positive gene expression was significantly lower than the proportion of malignant mammary tumours which had positive gene expression (all p values < 0.05).

5.4 Discussion

All the chemokines and chemokine receptors analysed in the present study, except CXCL10 and CCR4, had higher gene expression in malignant CMGTs that subsequently metastasised than in malignant CMGTs that did not metastasise. In addition, higher expression of CXCR4, CCR9, CCL5 and CXCL12 was associated with shorter survival times of the dogs. Of the tested chemokines and chemokine receptors, CCL5 was identified to predict the survival times of the dogs with malignant mammary neoplasms independent of tumour size, histological grade, presence of tumour emboli in histological sections and gene expression of other included chemokines and chemokine receptors. Therefore, the present findings suggest that the expression of chemokines and chemokine receptors influences tumour behaviour. To the author's knowledge, this is the first time that chemokine and chemokine receptor gene expression in CMGTs has been associated with disease outcome in dogs.

The present results are consistent with many human breast cancer studies which have also revealed expression of chemokines and chemokine receptors by neoplastic cells to

influence tumour behaviour and patient survival.[15,17,18,21,13,23,36] These human studies have identified several mechanisms to explain how the expression of chemokines or chemokine receptors may influence tumour behaviour.[3] Firstly, the chemokine network in a tumour has been shown to influence the extent and phenotypic composition of the inflammatory cell infiltrate within the tumour. Some inflammatory cells are pro-tumourigenic while others have anti-tumourigenic properties.[3,5] Therefore, altered chemokine gene expression may indirectly influence the tumour behaviour by changing the intra-tumoural inflammation in a way that promotes tumour growth and spread. Secondly, aberrant expression of chemokine receptors on the surface of neoplastic cells can promote migration of these cells towards distant organs that contain higher concentration of the corresponding chemokine.[3] For example, human breast cancers with high CXCR4 expression were shown to metastasise to lung, liver and bone more frequently than breast cancers with low CXCR4 expression. This is hypothesised to be because lung, liver and bone tend to contain a high concentration of CXCL12 which binds to the CXCR4 receptors.[3]

The use of chemokine expression to predict prognosis has been investigated for many different human cancer types.[12,1,25] For example, chemokine panels consisting of 7 to 12 chemokines were shown to accurately predict the behaviour of breast cancers.[28,34] In dogs, it is difficult to accurately predict the behaviour of malignant mammary neoplasms using existing conventional prognostic tools.[32] The inability to predict which neoplasms are likely to metastasise can delay the therapeutic interventions aimed at preventing mammary tumour metastasis. In the present study, relative expression of chemokines CCL5 and CXCL12 was significantly higher in mammary tumours which subsequently developed metastasis compared to the tumours which did not develop metastasis. Chemokine CCL5 was also identified to predict the survival times of the malignant canine mammary neoplasms independent of conventional prognostic indicators. In addition, chemokine receptors CXCR3, CXCR4, CXCR7 and CCR9 were detected more frequently in the tumours that subsequently metastasised compared to those which did not develop clinical evidence of metastasis. Therefore, the present findings suggest these chemokines and chemokine receptors may be useful to predict which CMGTs are more likely to metastasise. However, while measuring the expression of chemokines and chemokine receptors may help to predict prognosis, the methods are technically challenging and additional method

development would be required before adopting measuring gene expression as a routine diagnostic tool.

As well as their potential use as prognostic markers, chemokines and chemokine receptors represent potential targets for cancer immunotherapy.[35,19] For example, if increased chemokine receptor activity leads to a more aggressive clinical behaviour of a neoplasm, blocking this receptor using a chemokine antagonist or a monoclonal antibody could reduce the likelihood of tumour metastasis and metastasis related death.[3,19] Recently, a monoclonal antibody and several synthetic and natural chemokine receptor antagonists have been approved by the United States Food and Drug Administration to treat several human cancers including some types of breast cancers.[19] However, considering the extent of research being carried out in the field of chemokine-targeted cancer immunotherapy, only a limited number of chemokine-targeted novel therapeutics have been introduced for clinical use. The lack of an appropriate animal model which mimics the characteristics and behaviour of human breast cancers contributes to the difficulties in developing novel chemokine related target therapies.[19] Recently, dogs have been proposed as a better animal model than mouse or rat models for pre-clinical testing of cancer therapeutics.[27,24] The findings of the present study show that chemokine and chemokine receptor gene expression patterns in CMGTs resemble those of human breast cancers. Additionally, these results show that expression of chemokines and their receptors influence the behaviour of mammary gland neoplasms in dogs. Therefore, mammary gland neoplasms in dogs may provide an appropriate animal model of human breast cancers for testing chemokine-targeted cancer therapeutics. Furthermore, the similarity of chemokine expression between CMGTs and human breast cancers may suggest the possibility of using already available chemokine receptor-targeted cancer therapeutics intended for human use to treat CMGTs.

In the present study, gene expression of CXCR3, CXCR4, CXCR7, CCR9, CCL5 and CXCL12 was associated with tumour behaviour similar to studies of human breast cancers. In contrast, expression of CXCL10 and CCR4 has been shown to predict tumour behaviour in humans but was not significantly associated with metastasis or survival time in the present study of CMGTs. The reason for this difference was unclear; however, only a relatively

small number of samples were able to be analysed for CXCL10 due to inappropriate melting temperatures observed in some samples. The smaller than anticipated sample size may have contributed to the lack of significant findings observed for this chemokine. It is also possible that the mechanism by which CXCL10 and CCR4 influence the behaviour of human cancers does not apply to CMGTs due to differences in tumour biology between dogs and humans.

The present study did not identify any significant differences in chemokine gene expression between different histological types and histological grades of malignant CMGTs. Although it is possible that chemokine expression in CMGTs is not affected by histological type and grade, it is necessary to further investigate this matter with a larger sample size including sufficiently high number of samples in different histological types and tumour grades.

Similar to the prognostic study described in chapter 4, in this study also tumour histological grade was identified to be independently prognostic of survival times of the dogs with malignant CMGTs while tumour size or presence of intra-vascular or intra-lymphatic tumour emboli were not independently predictive of metastatic behaviour of malignant CMGTs. As described in the discussion of chapter 3 of this thesis, these findings were consistent with previous studies.[29, 14, 26,32,9]

A significantly lower proportions of benign CMGTs had positive gene expression for chemokine and chemokine receptor genes in this study. This is consistent with previous canine studies and indicated that expression of chemokine and chemokine receptor genes increases as differentiation of a neoplasm decreases.[2,6] However, the ideal comparison of chemokine and chemokine receptor expression should be performed between neoplastic mammary gland and the non-neoplastic mammary gland adjacent to the neoplastic mammary gland. This could not be fulfilled in the present study due to insufficient non-neoplastic mammary gland available in the tissue sections and therefore benign mammary tumours were used as an alternative to non-neoplastic mammary gland. Although the benign mammary neoplasms are closely representative of the chemokine expression of the non-neoplastic mammary gland, more studies are necessary to confirm these findings.

One limitation of the present study was the lack of tumour staging at diagnosis. This suggests it is possible that some CMGTs could have metastasised prior to the neoplasm being excised. Additionally, due to the retrospective nature of the study, it was impossible to definitively rule out the possibility that some of the CMGTs that were classified as non-metastatic had developed clinically silent metastatic disease in the 3-year period of the study.

Except for three benign mammary gland tumours, all of the mammary neoplasms contained an inflammatory cell infiltrate which was predominantly present in the tumour stroma. Most of these inflammatory cells were not included for RNA extraction as the stromal tissues were removed prior to RNA extraction. However, absolute exclusion of inflammatory cells or tumour stromal tissues from RNA extraction was not possible and this is recognised as a limitation of the present study. Further studies using immunohistochemistry are needed to confirm that chemokines and chemokine receptor proteins are expressed in neoplastic cells and to identify which neoplastic cells produce them in tumours where more than one cell type exists such as mixed mammary tumours.

In summary, the present study is the first reported investigation of chemokine and chemokine receptor gene expression in CMGTs with known clinical outcome. The results showed that expression of some of the chemokine and chemokine receptor genes was significantly associated with the development of metastases and survival times in these dogs. As well as providing insight into the factors that influence behaviours of CMGTs, these results suggest that chemokines and chemokine receptors may have future uses as prognostic markers or therapeutic targets in these common life-threatening neoplasms of dogs.

5.5. Bibliography

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Chapter 6 : Prognostic significance of immune checkpoints PD-L1 and CTLA-4 in canine mammary gland tumours

6.1 Introduction

The two previous chapters described the prognostic significance of cancer-associated inflammation related prognostic markers including stromal mast cell density, and chemokines and chemokine receptors. In addition to these cancer-associated inflammation related markers, many studies have shown that expression of immune checkpoint molecules by cancer cells influence the behaviour of human breast cancers and thereby are useful as prognostic markers. This chapter presents the outcome of the investigations carried out to determine whether the two immune checkpoint molecules: programmed death ligand-1 (PD-L1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) which were shown to be prognostic of human breast cancer behaviour are similarly prognostic for canine mammary gland tumours (CMGTs) .

Immunosurveillance to detect and subsequently destroy infected or abnormal cells is a major function of the immune system. The activation of T-cells is an important event in immunosurveillance. This activation occurs following interactions between T-cell receptors and major histocompatibility complex proteins that are present on the surface of antigen presenting cells.[14] However, T-cell activation is also controlled by a group of signalling molecules collectively referred to as immune checkpoints, which are typically comprised of receptors located on T-cells that interact with a corresponding ligand present on antigen presenting cells.[14,21] Immune checkpoints can either be activating whereby activation allows T-cells to proliferate or inhibitory whereby receptor activation suppresses T-cell activation.[21,14,10]

Recent research has shown that many human cancers aberrantly express inhibitory immune checkpoint molecules which suppress T-cell activation.[10,14] This suppression of T-

cell activity may allow neoplasms that express inhibitory immune checkpoint molecules to evade host immune surveillance resulting in a more aggressive clinical behaviour and less responsiveness to treatments. Two inhibitory immune checkpoints that are often expressed in human cancers are PD-L1 and CTLA-4.[21,47] Studies have demonstrated that neoplasms with increased PD-L1 and CTLA-4 have a more aggressive behaviour and a worse disease outcome, therefore measuring these immune checkpoint molecules could be a useful way to predict prognosis.[30,45] Further, using monoclonal antibodies (mAbs) to block these immune checkpoints has been shown to enhance cytotoxic T-cell mediated tumour cell destruction as well as increasing the activation of other immune responses such as antigen presentation and cytokine release.[6,45] Currently, several such anti-PD-L1 and anti-CTLA-4 mAbs are available to treat a variety of human cancer types.[45]

In veterinary medicine, several previous studies have shown that a variety of canine cancers express PD-L1 and CTLA-4.[2,22,26,41] The expression of PD-L1 and CTLA-4 on neoplastic lymphoid cells was previously shown to predict the prognosis of canine high-grade B cell lymphomas.[2] Further, *in vitro* studies have shown that PD-L1 blockade increases cytokine release by cultured cancer cells[25] and anti-PD-L1 mAbs have been administered to a small number of dogs with cancer.[27] Additionally it was shown that PD-L1 protein is more frequently detectable in histologically-malignant CMGTs than histologically-benign ones.[26,41] However, whether evaluation of this protein can predict the biological behaviour of CMGTs has not been previously evaluated. To the author's knowledge, there have been no studies of CTLA-4 in CMGTs. Therefore, the present study investigated the immunostaining and gene expression of PD-L1 and CTLA-4 in 41 malignant and 12 benign CMGTs. As the clinical outcome of these tumours was known, it could be determined whether PD-L1 and CTLA-4 protein and gene expression were correlated to CMGT behaviour and clinical outcome.

6.2 Material and methods

6.2.1 Case selection and assessment of survival times

The same set of canine mammary tumour cases described in detail in Chapter 4 was used for this study. Therefore, the case selection procedure and the inclusion criteria were the same as in sections 4.2.1 in Chapter 4. Briefly, mammary gland tumour cases submitted to IDEXX diagnostic laboratory, New Zealand, for histopathology between 2012 and 2015 were sourced from the laboratory surgical biopsy archive. Details regarding the patient signalment were identified from the surgical biopsy archive data base while the clinical records of the patients and information regarding the post-surgical follow-ups were obtained by contacting the submitting veterinarians. Cases were excluded if adjunct therapies including anti-inflammatory drugs, steroids or anti-cancer drugs were used to alter the neoplasm behaviour or if the tumour surface was reported to be ulcerated or contained abscesses. The disease-specific survival time for each case was calculated retrospectively from the date of tumour excision to the date of the dog's death or euthanasia due to clinically-diagnosed mammary tumour metastasis.

6.2.2 Histological classification and grading

The histological classification and grading of the canine mammary neoplasms was performed as described in section 4.2.2 in Chapter 4 following Goldschmidt et al. (2011) and Pena et al. (2013) classifications.[11,34] Tumours were classified into three groups; malignant-metastatic, malignant non-metastatic and benign following the criteria described in the same section of Chapter 4.

6.2.3 Immunohistochemistry

6.2.3.1 Anti-PD-L1 and anti-CTLA-4 antibody validation

The two primary antibodies used for PD-L1 and CTLA-4 immunostaining in CMGTs were prepared against the corresponding human proteins. The anti-PD-L1 antibody (ab233482, Abcam, MA, USA) was raised against a peptide sequence between residues 60-100 which

has an 83% sequence similarity with canine PD-L1 protein. Similarly, anti-CTLA-4 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was raised against a peptide sequence included in the C-terminus of the human CTLA-4 protein which has an 84% sequence identity with the canine CTLA-4 protein. Therefore, before using them for immunostaining in CMGTs, the cross reactivity of these antibodies with the corresponding canine proteins were assessed by immunoblotting.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Mammary tumour tissues of a dog that died due to tumour metastasis were collected during a post-mortem examination and used for protein extraction. To extract protein from tissues, the mammary tumour tissues from the dog was minced into fine pieces and homogenised on ice. A protease inhibitor cocktail was then added to the tissue homogenate and proteins were extracted using ReadyPrep™ kit following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). The protein concentration of the extract was estimated using a commercial kit following manufacturer's instructions (Qubit™ Protein Assay Kit, Invitrogen, Carlsbad, CA, USA) and adjusted to 2 µg/µl. The protein extract was then mixed with an equal volume of 2X Laemmli sample buffer (Bio-Rad), and denatured by mixing with 2- mercaptoethanol (Bio-Rad) to have a 5% final concentration and then heat treated at 100°C for 10 minutes. Purified recombinant Fc Tag canine PD-L1 protein (Sino Biological, Wayne, PA, USA) was used as the positive control and prepared similarly to the protein extracted from mammary tumour tissues except adjusting the final protein concentration to 0.1 µg/µl. The processed samples were cooled to room temperature and 40 µl of each sample and the positive control were loaded into separate wells in a 4-20% SDS-PAGE gel (Criterion™ TGX™ Precast Midi Protein Gel, 12+2 well, 45 µl, Bio-Rad) along with a protein molecular weight marker (Precision Plus Protein™ Kaleidoscope™ Pre stained Protein Standards, Bio-Rad). The loaded gel was placed inside a protein electrophoresis apparatus (Criterion™ Vertical Electrophoresis Cell, Bio-Rad), filled with 1X running buffer (10x Tris/Glycine/SDS, Bio-Rad), and proteins were separated by running the apparatus at 100V for 1 hr. Once complete, the gel was separated, stained with Coomassie blue staining solution (Bio-Rad) and imaged to visualise the size of the resulting bands in both control and test samples.

Immunoblotting

The proteins were separated by electrophoresis as described previously. A working solution of transfer buffer was prepared by mixing 100 ml of 10X transfer buffer (10x Tris/Glycine Buffer for Western Blots and Native Gels, Bio-Rad) with 200 ml methanol and 700 ml distilled water. Polyvinylidene difluoride (PVDF) membrane (Immun-Blot® PVDF Membrane, Precut, 10 x 15 cm, Bio-Rad) was soaked in methanol for 1 minute and rinsed in 1X transfer buffer. A transfer sandwich was prepared by arranging following items in the given order ensuring no air bubbles between layers: plastic plate, sponge, filter paper, SDS-PAGE gel, PVDF membrane, filter paper, sponge and plastic plate. The prepared transfer sandwich was placed in a protein transfer apparatus (Criterion™ Blotter with wire electrodes, Bio-Rad) and transfer buffer was added to the to the apparatus until the sandwich was covered with buffer. An ice pack was placed in the front compartment of the apparatus to maintain 4°C during the transfer. The electrodes were placed over the transfer sandwich and run at 100V for 1 hour to transfer the proteins from the gel to the PVDF membrane. At the end of the transfer, the PVDF membrane was carefully retrieved from the sandwich, stained with Ponceau-S (Sigma-Aldrich, New South Wales, Australia) to visualise the successful protein transfer, rinsed with TBS with 0.1% Tween 20 (TBST) (Bio-Rad) and put in TBST with 5% non-fat dry milk (NFDM) for 1 hour to block non-specific binding. Next the PVDF membrane was rinsed with TBST three times and incubated with primary antibody (anti-human PD-L1 antibody, Rabbit polyclonal, ab233482, Abcam) diluted 1:100 in TBST with 5% NFDM and incubated overnight at 4°C. At the end of incubation, the membrane was rinsed four times with TBST and then incubated with the secondary antibody (Goat Anti-Rabbit IgG, HRP conjugated, ab233482, Abcam) diluted 1:8000 in TBST with 5% NFDM, for 1 hr at room temperature. The membrane was then rinsed three times with TBST, and molecular weight ladder was marked using a chemiluminescent marker pen (WesternSure® Pen, LI-COR Biosciences, Lincoln, NE, USA). The HRP substrate was prepared by mixing equal volumes of Luminol buffer and enhancer buffer from a commercial kit (Immun-Star HRP Chemiluminescent Substrate Kit, Bio-Rad). The prepared substrate was added over the surface of the PVDF membraned, incubated for five minutes and imaged using C-DiGit ® Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA) following manufacturers' instructions.

To determine the binding specificity of the anti-CTLA-4 antibodies, immunoblotting was performed the same as for the anti-PD-L1 antibodies except that purified recombinant His-Tag canine CTLA-4 protein (Sino Biological, Wayne, PA, USA) was included as the positive control and mouse anti-human CTLA-4 monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:50 and HRP conjugated Goat Anti-mouse IgG antibody, (ab205719, Abcam) diluted 1: 8000 were used as the primary and secondary antibodies respectively.

6.2.3.2 Immunohistochemistry

Three micrometre thick sections of paraffin-embedded formalin-fixed (FFPE) canine mammary tumour tissues were pre-treated by a heat-induced antigen retrieval method using citrate buffer (0.01 M, pH 6.0). The primary antibody was a rabbit anti-PD-L1 polyclonal antibody (ab233482, Abcam) which was used at 1:100 dilution. Tissue sections were incubated with the primary antibody for 2 hours at room temperature. Antigen detection was performed using VECTASTAIN® Elite® ABC HRP kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions and the immunoreactivity was visualised using 3, 3' diaminobenzidine chromogen (Biocare Medical, Pacheco, CA, USA) with a haematoxylin counterstain. A cutaneous granuloma from a dog was used as the positive control while the primary antibody was omitted from the negative controls. Additionally, sections of adrenal gland and pancreas obtained from a dog that died of an unrelated cause were also used as negative controls as these tissues have been previously shown not to contain PD-L1 protein.[41]

The primary antibody used for CTLA-4 immunohistochemistry was a mouse anti-CTLA-4 monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) used in 1:50 dilution. The rest of the method used for immunostaining of CTLA-4 was the same as for immunostaining of PD-L1. A section of mandibular lymph node obtained from a dog that had died due to an unrelated cause was used as the positive control while the primary antibody was omitted for the negative control.

6.2.3.3. Quantification of immunostaining

Immunostaining was quantified using a method which considered both the staining intensity and the proportion of positive cells present in a high-power (400x) microscopic field. Briefly, the immunostaining intensity of tumour cells in a 400x microscopic field was assessed using a scale that ranged from 0-3. For PD-L1, both the membrane and cytoplasmic immunostaining were considered together when determining the immunostaining of a cell while only cytoplasmic immunostaining was considered for CTLA-4, consistent with previous reports on immunostaining patterns of these two proteins in canine and human tissues.[33,37,41] The 0-3 scale for PD-L1 was define as : 0 = membrane and cytoplasmic immunostaining is absent, 1 = incomplete membrane immunostaining with or without mild or moderate cytoplasmic staining, 2 = moderately intense complete membrane immunostaining, with or without moderate cytoplasmic immunostaining, 3 = intense complete membrane immunostaining with or with or without moderate or intense cytoplasmic immunostaining. The scale for CTLA-4 was defined considering cytoplasmic immunostaining: 0 = cytoplasmic immunostaining is absent, 1 = mild cytoplasmic staining, 2 = moderate cytoplasmic immunostaining, 3 = intense cytoplasmic immunostaining To generate an immunostaining score, the percentage of immunostaining positive cells identified in each level of the scale were applied to the following formula: immunostaining score = 0 x % of level 0 cells + 1 x % of level 1 cells + 2 x % of level 2 cells + 3 x % of level 3 cells. The lowest possible immunostaining score was 0 while the highest was 300. From each tissue section, five representative high-power fields were individually scored, and the average was considered as the final immunostaining score for that mammary tumour. In instances where more than one tissue block was available for a single tumour, the average of the immunostaining scores calculated for different sections was taken as the final immunostaining score for the tumour. For the dogs which had multiple mammary neoplasms, the malignant neoplasm was used for immunostaining. Additionally, nuclear immunostaining of PD-L1 and CTLA-4 immunostaining status of the tumour infiltrating immune cells was also recorded.

6.2.4 Real time PCR (RT-PCR) for PD-L1 and CTLA-4 genes

RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted from FFPE canine mammary tumour samples using Nucleospin totalRNA FFPE XS kit (Macherey-Nagel, Düren, Germany) following manufacturer's instructions and nucleic acid concentrations were quantified. Three, 10 µm sections from each block were used for RNA extraction. When multiple FFPE blocks were available for a single tumour, RNA extraction was performed from each block separately and the extracts were mixed prior to further assessments. To remove any residual DNA, post-extraction DNAse digestion was performed using Ambion Turbo DNA-free DNAse following the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). When a single dog had multiple tumours, the malignant tumour was used for RNA extraction. RNA was extracted from a mandibular lymph node collected from a dog that had died of an unrelated cause to be used as positive controls in PD-L1 and CTLA-4 RT-PCR assays.

Complementary DNA (cDNA) was synthesised from the total RNA extracted from canine mammary tumour samples using Transcriptor first-strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany). Primers for PD-L1 and CTLA-4 were developed using the reference sequences of the target canine genes available in NCBI GenBank and Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and mfold (http://unafold.rna.albany.edu/?q=mfold) algorithms. All RT-PCR assays were performed in a Mic qPCR Cycler (Bio Molecular System, Upper Coomera, Australia). A 10 µL reaction mix included, 10 ng of cDNA, forward and reverse primers in 0.7 µM concentration for PD-L1 and 0.5 µM for CTLA-4 and AccuMelt HRM SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). The following running conditions were used for RT-PCR assays for PD-L1: :denaturation at 95 °C for 5 minutes, followed by 40 cycles at 95 °C for 30 seconds, 55 °C for 15 seconds and 70 °C for 20 seconds. For CTLA-4 running conditions included denaturation at 95 °C for 5 minutes, followed by 40 cycles at 95 °C for 30 seconds, 57 °C for 15 seconds and 70 °C for 20 seconds. All RT-PCR assays for mammary tumour samples were performed in duplicate and each plate included a positive control and a no template control. The residual genomic DNA was excluded based on the melting temperature and/or minus-RT controls. Reference gene stability was analysed using GeNorm application in

qbase+ software (qbase+, Biogazzele, Gent, Belgium). The fold change in the mRNA expression in malignant mammary tumours was calculated by $2^{-\Delta\Delta CT}$ method using the data obtained from PD-L1 and CTLA-4 RT-PCR assays. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and ribosomal protein L32 (RPL-32) were used as the reference genes and the primers for these genes were obtained from previous publications.[5,24] All the primers used in this study are included in Table 6.1.

Validation of PD-L1 and CTLA-4 RT-PCR assays

The newly developed PD-L1 and CTLA-4 assays were validated by determining the sensitivity, precision, reproducibility, and specificity of the assays. Sensitivity of the assays was determined using three ten-fold serial dilution assays of the amplicon while the linearity of the assays was determined from the efficiency and r^2 values calculated using the curves generated from the dilution assays. Precision was evaluated by calculating the intra-assay variability based on the distribution of linearized Ct values for five replicates of each standard in a single PCR run. Reproducibility was evaluated by calculating inter-assay variability comparing the linearized Ct values obtained for the same standards in the three separate dilution assays. The intra- and inter-assay variability was expressed as a coefficient of variance (CV), which was the ratio of the standard deviation to the mean of the linearized Ct values. To assess the specificity of the assays the products amplified with the PD-L1 and CTLA-4 primers were purified and sequenced. Briefly, the PCR products were digested with Shrimp Exonuclease I and Alkaline phosphatase enzymes (illustra ExoProStar, Life Sciences, St. Petersburg, FL, USA), mixed with 4 nmoles of forward or reverse primers and sent to the DNA sequencing facility of Massey University, NZ.

Table 6.1 Primers used for PD-L1 and CTLA-4 RT-PCR assays.

Gene	NCBI Accession No:	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product length (bp)
PD-L1	XM_005615937.2	CCAGCAGGTCACTTCAGAAC	CCATTGTCACATTGCCACCA	136
CTLA-4	NM_001003106.1	CCCCGTCTTCTCCAAAGGGAT	TATGTCGCGGCACAGACTTC	174
HPRT	AY283372	AGCTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC	114
RPL32	XM_848016.1	TGGTTACAGGAGCAACAAGAA	GCACATCAGCAGCACTTCA	100

6.2.5 Statistical analysis

Kruskal-Wallis H test was used to compare the PD-L1 and CTLA-4 immunostaining scores and gene expression between malignant-metastatic and malignant non-metastatic CMGTs. The correlation between PD-L1 or CTLA-4 immunostaining scores and corresponding gene expression was tested using Spearman rank-order correlation test. The same test was used to evaluate the correlations between PD-L1 or CTLA-4 immunostaining scores and survival times of the dogs with malignant mammary gland tumours. X-tile software (https://medicine.yale.edu/lab/rimm/research/software/)[7] was used to identify appropriate cut-off points to classify malignant mammary neoplasms into PD-L1 or CTLA-4 immunostaining "high" and "low" categories based on the disease outcome of the patients. A hierarchal multivariate analysis was performed to identify whether "high" immunostaining scores of PD-L1 or CTLA-4 were predictive of shorter disease specific survival time in dogs with malignant mammary neoplasms independent of conventional prognostic factors including tumour size, tumour grade and presence of intra-vascular or intra-lymphatic tumour emboli in histological sections. Pearson Chi-Squared test was used for group comparisons regarding nuclear immunostaining of PD-L1 and immunostaining positivity of tumour infiltrating immune cells for PD-L1 or CTLA-4. All statistical analyses, except cut-off analysis were performed using the SPSS version 25 program (IBM Corporation, Armonk, New York).

A final hieracheal multivariate analysis was also performed to identify which of the inflammation related prognostic markers investigated in this thesis were independently prognostic of survival times of dogs with malignant mammary neoplasms. This analysis included conventional prognostic factors of tumour size, tumour grade and presence of tumour emboli, along with the inflammation related prognostic markers of stromal mast cell density, CXCL12 and CCL5 gene expression and PD-L1 immunostaining.

6.3 Results

6.3.1 Selected cases

The clinicopathological characteristics of the selected patients are described in section 4.3.1 of Chapter 4.

6.3.2 Tumour size and histological classification

The characteristics of the tumours are described in section 4.3.2 of Chapter 4.

6.3.3 Disease outcome of the selected cases

The details of mammary tumour metastasis of the dogs included in the study are described in section 4.3.5 of Chapter 4. Briefly, 21/41 (51.2 %) dogs with malignant mammary tumours died after developing evidence of tumour metastasis within three years of surgical excision of the neoplasms (malignant-metastatic group). The other 20 dogs with malignant neoplasms were not diagnosed to have clinically evident mammary tumour metastasis during the 3-year follow-up period (malignant non-metastatic group).

6.3.4 SDS-PAGE and immunoblotting

The immunoblot developed using the anti-PDL1 antibody contained three distinct bands of approximately 34 kDa, 60 kDa and 150 kDa in the lane loaded with protein extracted from CMGT tissues (Figure 6. 1: B). There was a single band of approximately 150 kDa in the lane loaded with purified recombinant canine PD-L1 protein. Canine PD-L1 protein has 289 amino acids (https://www.uniprot.org/uniprot/?query=canine+PD-L1&sort=score) and the predicted molecular weight from the sequence is 33.1 kDa. The molecular weight of the canine PD-L1 was reported to be 35 kDa in a previous study and the band approximately of 34 kDa observed in the immunoblot of the present study was consistent with this previous

report.[22]Another study has reported canine PD-L1 molecular weight to be approximately 60 kDa and it was suggested that this might be due to the post transitional glycosylation.[31] The 60 kDa band observed in the immunoblot of the present study may represent a similarly modified PD-L1 protein. The higher molecular band around 150 kDa may correspond to a PD-L1 polymer. While multiple bands were observed, the results of the immunoblotting are consistent with satisfactory specificity for canine PD-L1 protein of the antibody used. This is supported by the lack of immunostaining visible in negative control tissues and the significant positive correlation detected between PD-L1 gene expression and PD-L1 immunostaining.

The immunoblot developed using the anti-CTLA-4 antibodies showed a single low molecular weight band of approximately 22 kDa and two higher molecular weight bands of approximately 200 kDa and 125 kDa in the lane which contained protein extracted from canine mammary tumour tissues (Figure 6.2: B). This band pattern was observed when proteins were denatured using mild reducing conditions which included heat treatment at 100 °C for 5 minutes and separating in gels containing SDS. However, when the proteins were denatured with severe reducing conditions, by addition of 2-mercaptoethanol, the intensity of these higher molecular weight bands decreased and the intensity of the lower molecular weight band increased, which may suggest the higher bands were comprised of polymerized proteins (Figure 6. 2: C). Only a single band of approximately 22 kDa was observed with the purified recombinant CTLA-4 protein (Figure 6.2: C).

The sequence of canine CTLA-4 protein contains 223 amino acids (https://www.uniprot.org/uniprot/Q9XSI1) and has an estimated molecular weight of 24.2 kDa. However, a soluble isoform of canine CTLA-4 (s-CTLA-4) which was shown to result from alternative splicing of CTLA-4 mRNA has been described previously.[43] The molecular weight of this s-CTLA-4 isoform is 23 kDa. The lower molecular weight bands observed in the CTLA-4 immunoblots of the present study were approximately 23 kDa and it was difficult to differentiate whether it corresponded to the splice variant or the full-length protein. The intensity of the two high molecular weight bands observed in the immunoblot decreased with a concurrent increase in the intensity of the lower molecular weight band, when the proteins were subjected to more intense denaturing conditions. Polymerisation

is not uncommon with human CTLA-4 and dimers and tetramers are frequently present in human immunoblots of this protein.[23,32] In addition, CTLA-4 has several glycosylation sites and so is prone to post translational glycosylation.[3] Therefore, the higher molecular weight bands are likely to be polymers of canine CTLA-4 which got denatured when more intense denaturing conditions were applied, although glycosylation is also a possibility. Overall, the immunoblotting suggests adequate specificity of the antibody used for canine CTLA-4 protein. As with PD-L1 this was supported by the correlation between CLTA expression and CLTA immunostaining within the sections.

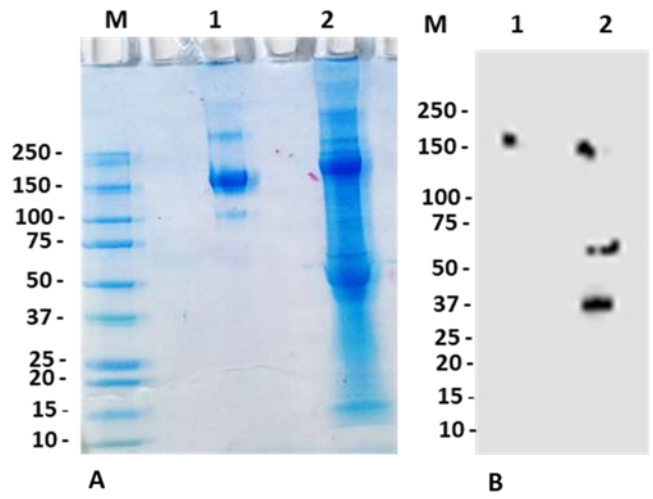

Figure 6.1 SDS-PAGE gel and corresponding immunoblot with anti-PD-L1 antibodies.

A: SDS-PAGE Gel, B: Immunoblot. M: Molecular weight marker (kDa), Lane 1: Purified recombinant canine PD-L1 protein-Fc tag (2 µg), Lane 2: Protein extracted from frozen mammary tumour tissues (40 µg). Primary antibody: rabbit anti PD-L1 polyclonal, 1:100 (ab233482), Secondary antibody: goat Anti-Rabbit IgG (HRP), 1:8000 (ab233482). Immunoblot exposure time: 6 minutes.

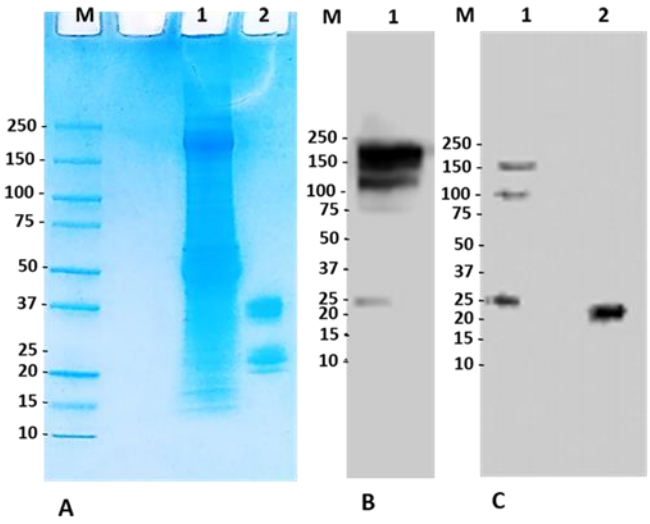

Figure 6.2. SDS-PAGE gel and corresponding immunoblot with anti-CTLA-4 antibody.

A: SDS-PAGE Gel. B: Immunoblot with proteins processed under mild denaturing conditions. C: Immunoblot with proteins processed with severe denaturing conditions. M: Molecular weight marker (kDa), Lane 1: Protein extracted from fresh mammary tumour tissues (40 µg), Lane 2: Purified recombinant canine CTLA-4 protein-Fc tag (2 µg). Primary antibody: mouse anti CTLA-4 monoclonal, 1:50 (Santa-cruz (F8) sc-376016). Secondary antibody: goat anti-mouse IgG (HRP), 1:8000 (ab205719). Immunoblot exposure time: 6 minutes.

6.3.5 Immunohistochemistry

Positive control samples immunostained as expected, with membrane and cytoplasmic immunostaining of PD-L1 observed in macrophages in the cutaneous foreign body granuloma that was used as the positive control (Figure 6.3). Immunostaining was absent in sections of adrenal gland and pancreas that were included as negative controls as well as the no-primary antibody control. The lymphocytes of the mandibular lymph node showed cytoplasmic immunostaining for CTLA- 4 while no immunostaining was present in the lymph node section prepared without the anti-CTLA-4 primary antibody (Figure 6.4). The 0–3 scale used for PD-L1 immunostaining quantification is depicted in Figure 6.5, and the scale for CTLA-4 in Figure 6.6.

One malignant CMGT was excluded from PD-L1 immunostaining analysis as inadequate tumour tissue was present on the section for accurate quantification. The PD-L1 immunostaining scores of the 40-remaining malignant CMGTs ranged from 6.1–286.5/HPF with a median of 82.5/HPF ± 124.4 (Q3-Q1). The median PD-L1 immunostaining scores of malignant tumours which subsequently metastasised (180.2/HPF ± 152.4 Q3-Q1) was significantly higher than those of malignant CMGTs that did not metastasise during the follow-up period (62.6/HPF ± 62.4 Q3-Q1, $p = 0.005$, Kruskal-Wallis H test). Two malignant mammary gland tumours were excluded from CTLA-4 immunostaining analysis due to inadequate tumour tissues to enable accurate quantification within the sections. In the remaining 39 malignant CMGTs, CTLA-4 immunostaining scores ranged from 40.8 – 281.8/HPF with a median of 170.4/HPF ± 122.9 (Q3-Q1). The CTLA-4 immunostaining score of the malignant mammary tumours which subsequently developed metastases (211/HPF ± 87.5 Q3-Q1) was significantly higher than the immunostaining scores of mammary tumours which did not develop tumour metastasis (144.3/HPF ± 100.9 Q3-Q1, $p = 0.003$, Kruskal-Wallis H test). Immunostaining for PD-L1 was observed in the nuclei of neoplastic mammary tumour cells in 11 of the 40 malignant mammary tumours.

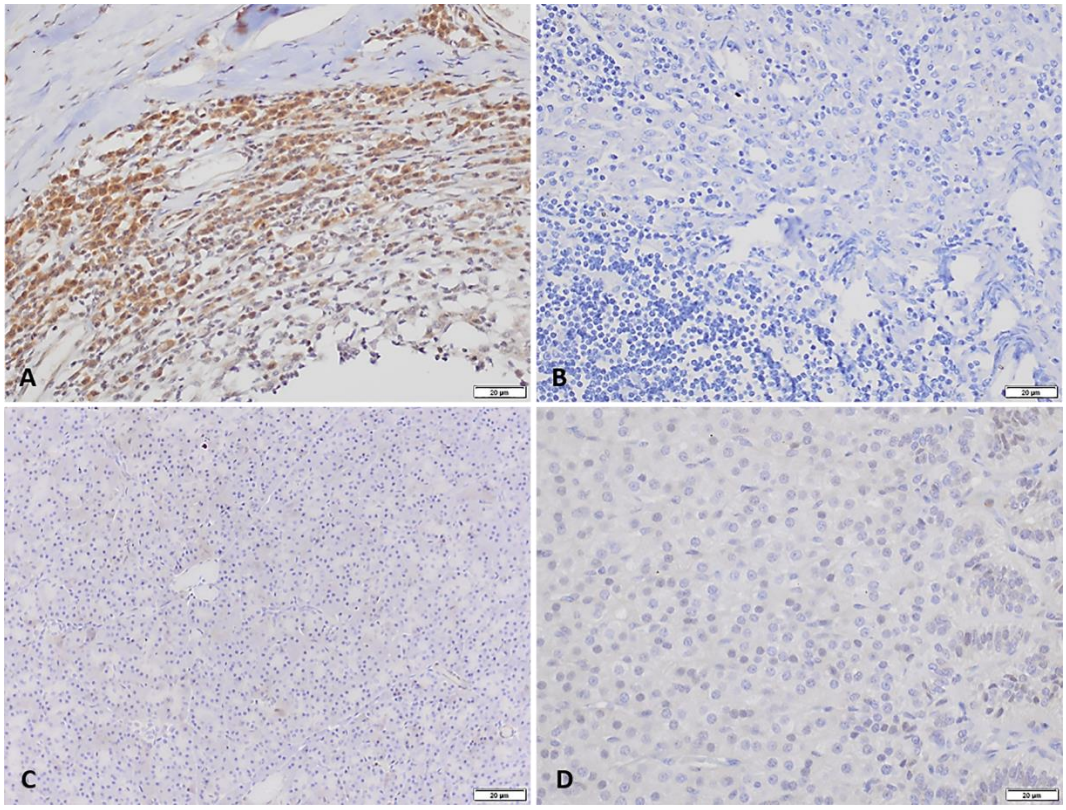

Figure 6.2 PD-L1 Immunostaining (primary antibody dilution 1:100).

A: Positive control – canine cutaneous foreign body granuloma. Note the positive staining in macrophages. B: Negative control –. cutaneous foreign body granuloma processed without primary antibody. C, D – additional negative controls, C: pancreas and D: adrenal gland of a dog. Bar = 20 µm.

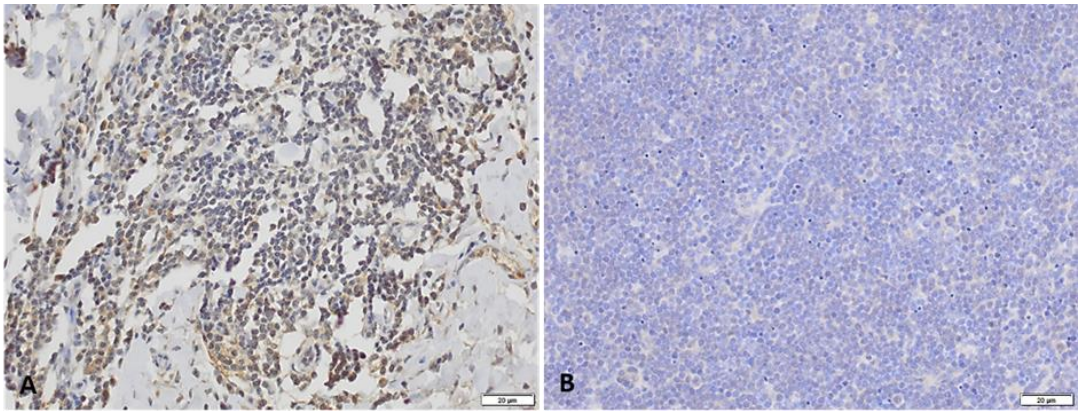

Figure 6.3 CTLA-4 immunostaining (primary antibody dilution 1:50).

A: Positive control – canine lymph node. Note the positive staining in lymphocytes. B: Negative control – canine lymph node processed without primary antibody. Bar = 20 µm.

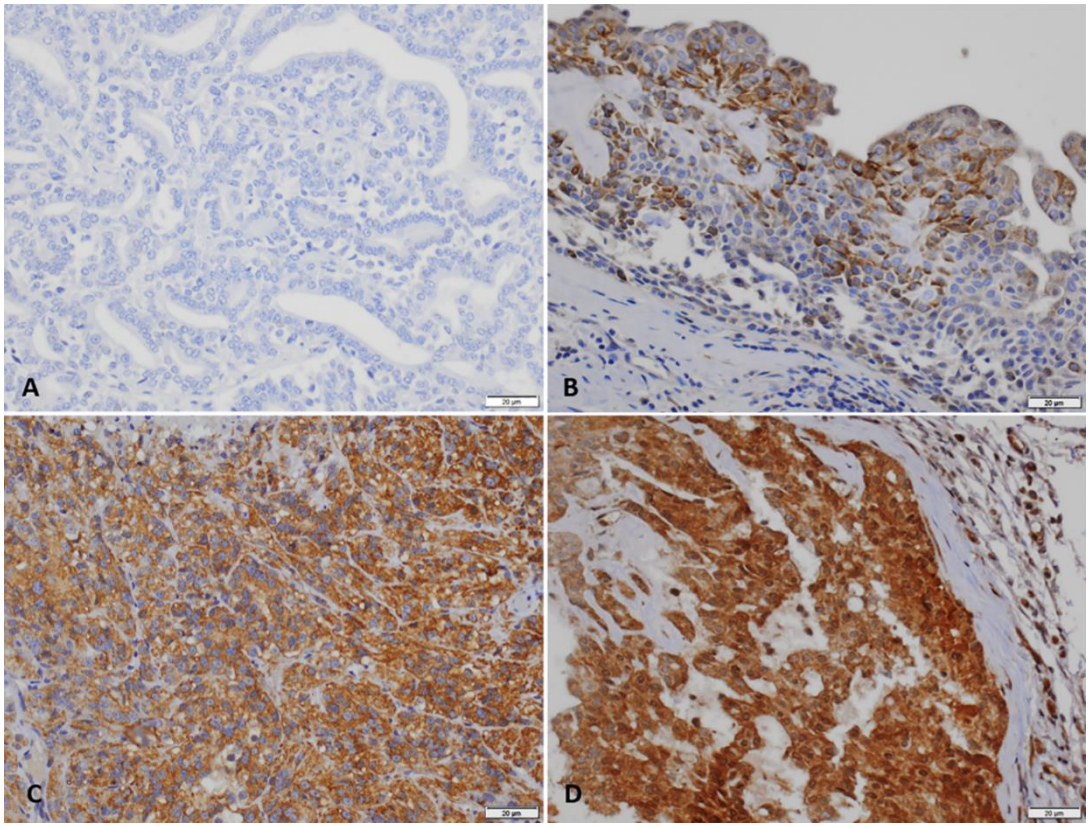

Figure 6.4 Canine mammary gland tumours with PD-L1 immunostaining (primary antibody dilution 1:100).

A: 0 = Absence of membrane or cytoplasmic immunostaining. B: 1 = Incomplete membrane immunostaining with or without mild or moderate cytoplasmic staining. C: 2 = Moderately intense complete membrane immunostaining, with or without moderate cytoplasmic immunostaining. D: 3: Intense complete membrane immunostaining with or without moderate or intense cytoplasmic immunostaining. Bar = 20 µm.

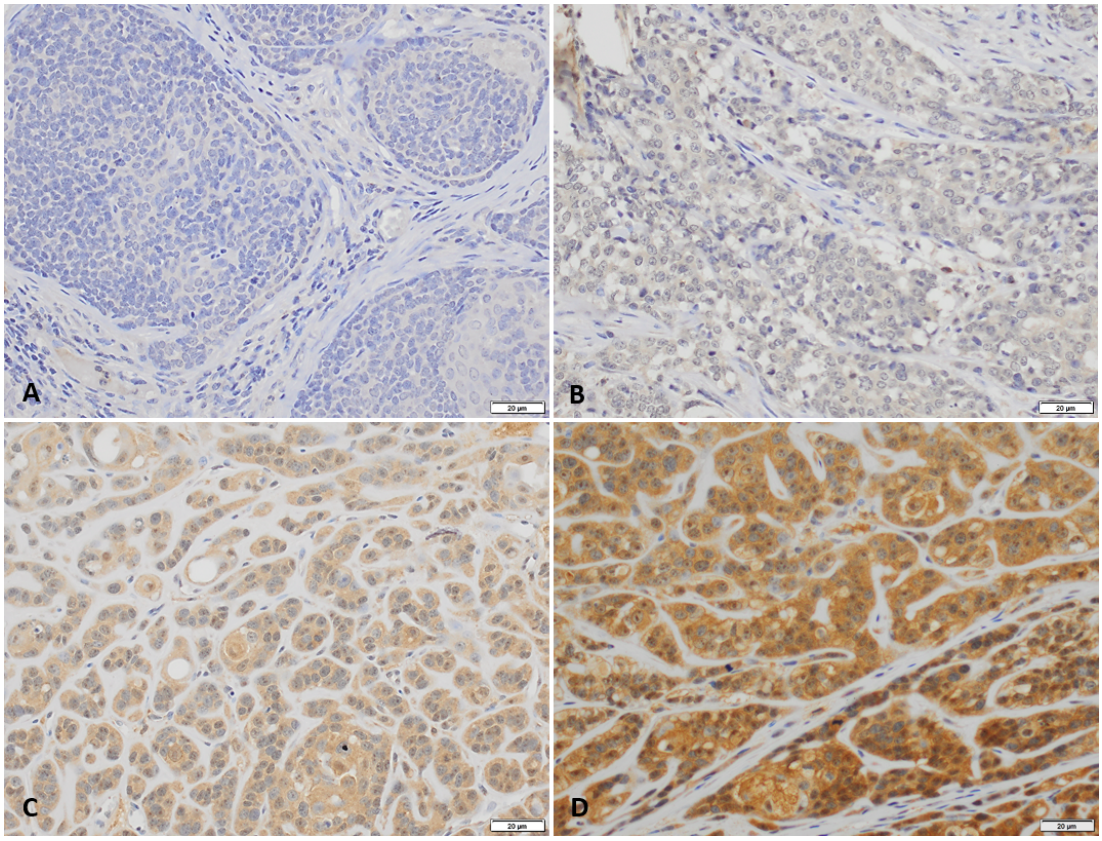

Figure 6.5 Canine mammary gland tumours with CTLA-4 immunostaining (primary antibody dilution 1:50).

A: 0 = Absence of cytoplasmic immunostaining. B: 1 = Mild cytoplasmic immunostaining. C: 2 = Moderate cytoplasmic immunostaining. D: 3 = Intense cytoplasmic immunostaining.

6.3.6 PD-L1 and CTLA-4 RT-PCR assay validation

The sequences of the PD-L1 and CTLA-4 amplicons produced using the newly developed primers showed 97.8% and 98.4% identity with the published cDNA sequences of canine PD-L1 and CTLA-4 (NCBI Blast search). The newly developed PD-L1 and CTLA-4 RT-PCR assays were linear within the tested range from 10^2 to 10^7 target copies and demonstrated adequate efficiency, r^2, and inter- and intra-assay precision and accuracy within the specified range (Table 6.2). The reference genes, HPRT and RPL32, showed adequate stability with a GeNorm M value ≤ 0.2 and a GeNorm V value < 0.15.

There was a significant positive association between PD-L1 immunostaining scores and relative gene expression in malignant CMGTs (r_s = 0.75, Spearman rank-order correlation test). A similar association was observed between CTLA-4 immunostaining scores and relative gene expression (r_s = 0.69, Spearman rank-order correlation test).

Table 6.2 Efficiency, r^2 and inter- and intra-assay CVs of PD-L1 and CTLA-4 RT-PCR says.

Gene	Assay efficiency	r^2	CV	Copies/ul					
				1 x 10^7	1 x 10^6	1 x 10^5	1 x 10^4	1 x 10^3	1 x 10^2
PD-L1	0.90	0.99	Inter-assay	16.3	6.6	7.3	7	12.6	20.4
			Intra-assay	14.7	22.6	8.5	5.9	13.7	20.1
CTLA-4	0.93	0.99	Inter-assay	8.3	1.2	4.4	8.1	2.6	19.3
			Intra-assay	8.5	9.8	6	7.5	7.8	19.9

6.3.6 Relative quantification of PD-L1 and CTLA-4 gene expression in canine mammary gland tumours

Of the 41 malignant CMGTs included in the RT-PCR assays, reference gene expression was absent in one mammary tumour and therefore this tumour was excluded from further analyses. Of the 40 malignant tumours which had detectable reference gene expression, PD-L1 expression was detected in 39 tumours. The PD-L1 relative gene expression in

malignant CMGTs which had subsequently metastasised was significantly higher than in the non-metastatic malignant mammary tumours (p = 0.023, Kruskal-Wallis H test).

Of the 40 malignant CMGTs with detectable reference gene expression, CTLA-4 expression could not be evaluated in six mammary tumours because the available sample volume was insufficient in one tumour, another had an inappropriate melting temperature, and four samples had only one replicate in which expression was detected. In three samples, no target gene expression was detected but there was positive reference gene expression, suggesting a true absence of CTLA-4 gene expression. These latter three samples were included in the statistical analysis following a modified method. Briefly, the Ct value of these samples were presumed to be higher than the Ct value of the standard which provided the lowest limit of detection in the validation assay. Therefore, the Ct values of these samples were calculated by adding three cycles to the corresponding Ct value of the lowest limit of detection of the assay. Then for each sample, the ΔΔCt was calculated by taking the difference between newly calculated Ct value and average reference gene Ct.The relative gene expression of CTLA-4 was significantly higher in canine MGTs that developed metastases than the relative gene expression in MGTs that did not metastasise during the follow-up period (p = 0.022, Kruskal-Wallis H test).

6.3.7 Cut-off analysis for PD-L1 and CTLA-4 immunostaining scores.

By X-tile analysis, a strong, inverse and continuous association between PD-L1 immunostaining scores and survival times of the dogs with malignant mammary tumours was identified. Furthermore, the software identified a PD-L1 immunostaining score of 180/HPF ($\chi 2$ = 17.88, p < 0.001) as the optimal cut-off which best predicted the disease outcome. Therefore, a malignant CMGT with a PD-L1 immunostaining score >180/HPF is more likely to develop metastasis while a tumour with immunostaining score ≤180/HPF is less likely to metastasise. An inverse continuous association was identified between CTLA-4 immunostaining scores and disease outcome of the dogs with an optimal cut-off of

177/HPF ($\chi 2 = 7.6$, $p = 0.07$). This suggests that a malignant CMGT with a CTLA-4 immunostaining score >177/HPF is more likely to develop tumour metastasis while a tumour with an immunostaining score ≤177/HPF is less likely to metastasise.

6.3.8 Correlation between PD-L1 or CTLA-4 immunostaining scores and the survival times of the dogs with malignant mammary gland tumours and prognostic ability of PD-L1 and CTLA-4 immunostaining scores on survival times.

The overall mean survival time of the 41 dogs with malignant CMGTs was 721 days (95% CI 609–833). There was a significant negative correlation between PD-L1 immunostaining scores and the disease specific survival times of dogs with malignant mammary neoplasms ($r_s = - 0.42$, $p = 0.008$) suggesting that the higher the PD-L1 immunostaining score, the shorter the survival time. The correlation between CTLA-4 immunostaining scores and the survival times of dogs with malignant mammary neoplasms also was significant and negative (($r_s = - 0.4$, $p = 0.01$).

The hierarchal multivariate analysis showed that PD-L1 immunostaining scores ($\Delta F = 4.9$, $p = 0.035$) and tumour grade ($\Delta F = 5.1$, $p = 0.03$) were independently prognostic of survival times of dogs with malignant mammary neoplasms. In contrast, CTLA-4 immunostaining ($\Delta F = 1,7$, $p = 0.2$), tumour size ($\Delta F = 1,8$, $p = 0.18$), and the presence of tumour emboli in histological tumour sections ($\Delta F = 1.1$, $p = 0.3$) were not independently prognostic of survival time. Mammary tumour histological sub-type was not included in multivariate analysis due to inadequate number of samples in some categories.

6.3.9 PD-L1 and CTLA-4 immunostaining scores and gene expression in benign mammary gland tumours.

The PD-L1 immunostaining scores of the 12 benign tumours included in the study ranged from 0–110/HPF with a median immunostaining score of 10.8/HPF. The immunostaining scores of the benign tumours was significantly lower than those of the malignant CMGTs (p = 0.001, Kruskal-Wallis H test). The CTLA-4 immunostaining scores of the benign mammary tumours ranged from 0-119.5/HPF with a median score of 95/HPF. The immunostaining scores of the benign tumours was significantly lower than the immunostaining scores observed for malignant CMGTs (p = 0.006, Kruskal-Wallis H test). The PD-L1 and CTLA-4 immunostaining scores of CMGTs classified as benign, malignant non-metastatic and malignant-metastatic are shown in Figure 6.7 and Figure 6.8. Immunostaining for PD-L1 was observed in the nuclei of neoplastic mammary tumour cells in a single benign mammary gland tumour. Of the 12 benign mammary neoplasms, detectable PD-L1 gene expression was observed in a single neoplasm while CTLA-4 expression was observed in three neoplasms.

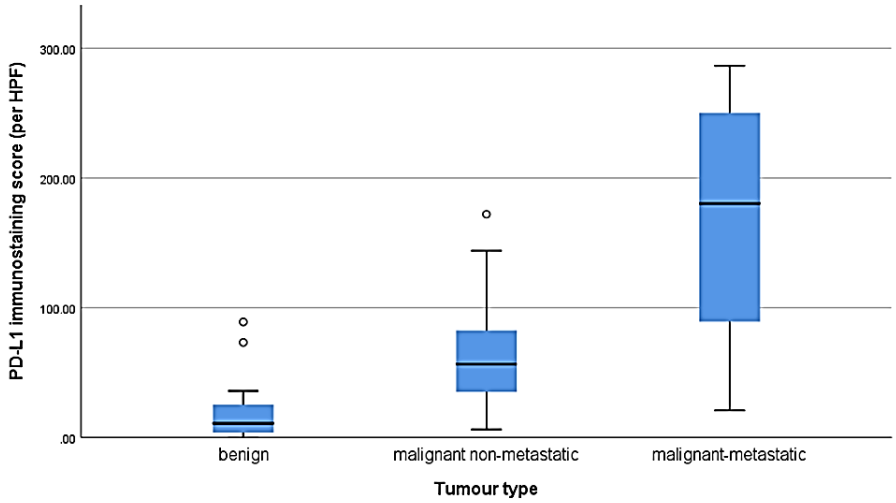

Figure 6.6 Boxplots of PD-L1 immunostaining in "malignant-metastatic", "malignant non-metastatic" and benign canine mammary gland tumours.

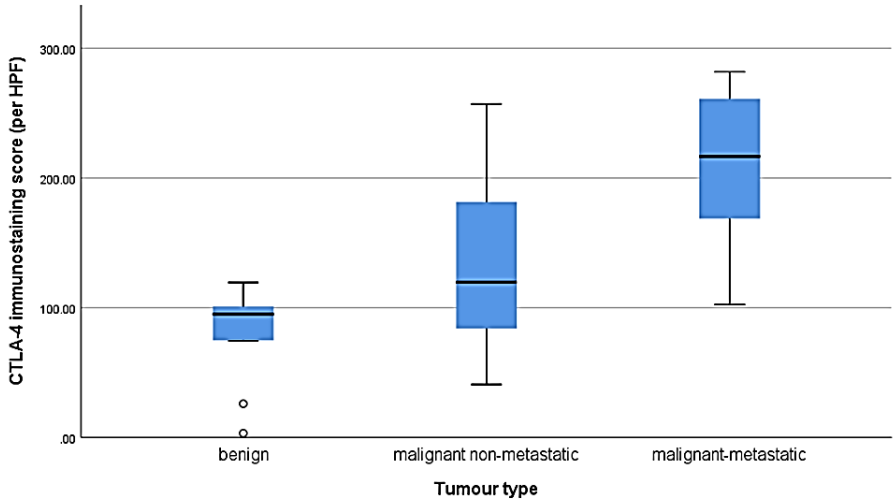

Figure 6.7 Boxplots of CTLA-4 immunostaining in "malignant-metastatic", "malignant non-metastatic" and benign canine mammary gland tumours.

6.3.10 Immunostaining of tumour infiltrating immune cells for PD-L1 and CTLA-4 in malignant canine mammary gland tumours.

All 41 malignant CMGTs included in the study had a population of tumour infiltrating immune cells which predominantly comprised of lymphocytes. Tumour infiltrating immune cells had PD-L1 immunostaining in 11 CMGTs. This included seven tumours which subsequently developed tumour metastasis which was not significantly higher than four tumours which did not metastasise (p = 0.22, Pearson Chi-squared test). The infiltrating immune cells present in all malignant mammary tumours included in the study showed CTLA-4 immunostaining.

Results of the final hieracheal multivariate analysis, including the tumour-associated inflammation related prognostic markers investigated in this thesis, revealed that tumour grade (ΔF = 4.9, p = 0.028), PD-L1 immunostaining (ΔF = 4.9, p = 0.042) and stromal mast cell density (ΔF = 4.9, p = 0.02) are independently prognostic of the survival times of dogs with malignant mammary neoplasms. None of the other prognostic markers were independently prognostic of survival times of dogs with malignant mammary neoplasms ($p > 0.05$).

6.4 Discussion

In the present study, the PD-L1 and CTLA-4 immunostaining scores of malignant CMGTs which subsequently developed tumour metastasis were significantly higher than the scores of malignant CMGTs that did not develop clinically evident tumour metastasis. In addition, higher PD-L1 and CTLA-4 immunostaining scores showed a significant association with shorter survival times of the dogs with malignant mammary neoplasms. These findings indicate that production of PD-L1 or CTLA-4 proteins by neoplastic canine mammary gland cells influences whether the cells from that neoplasm are likely to metastasise. To the author's knowledge, this is the first time that either PD-L1 or CTLA-4 have been associated with the clinical outcomes of mammary gland neoplasms in dogs. How the increased

immune checkpoint proteins influence tumour metastasis in CMGTs is not known. However, previous human studies have suggested that increased PD-L1 or CTLA-4 influences tumour metastasis by modulating the immune system. Generally, the immune system is protective against metastases and therefore suppression of the immune system through checkpoint molecules may promote tumour metastasis.[4,46] This is supported by the observation that immunosuppression facilitates some steps of the metastatic cascade including cancer cell exfoliation, survival in circulation, and establishment at distant sites.[13,39] Therefore, it appears likely that increased PD-L1 or CTLA-4 expression within a neoplasm may facilitate tumour metastasis by inhibiting the immune reaction against the neoplastic cells.

Consistent with the findings of the present study, studies of human cancers have also associated increased PD-L1 production by cancer cells with increased lymph node metastasis, shorter disease free times, and shorter overall survival times.[15,16,18,28] Due to this association between the presence of PD-L1 and poor disease outcome, PD-L1 has been proposed as a potential prognostic marker for these neoplasms. The findings of the present study suggest that PD-L1 immunostaining could be similarly useful to predict the prognosis of malignant CMGTs.

As with PD-L1, there were significant differences in immunostaining and gene expression of CTLA-4 between metastatic and non-metastatic CMGTs. This suggests that evaluation of CMGTs for this protein could also be useful to predict prognosis. In human medicine, despite the well-known immunosuppressive role of CTLA-4, the association between the expression of CTLA-4 protein by cancer cells and disease outcome is unclear, possibly due to the limited numbers of studies investigating this association.[40,48] While one study reported CTLA-4 immunostaining predicted prognosis independently of other conventional prognostic factors[40], it was not identified as an independent prognostic factor in another similar study.[48] In the present study, unlike PD-L1, CTLA-4 immunostaining was not identified to be independently prognostic of survival times of the dogs with malignant mammary neoplasms.

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In the present study, immunostaining scores for PD-L1 and CTLA-4 ranged from 0–300/HPF with neoplasms that metastasised having higher immunostaining scores than those that did not. For pathologists to be able to predict which tumours are likely to metastasise, it would be helpful to have a cut-off score which divides tumours into immunostaining "high" and immunostaining "low" categories. The cut-offs derived in the present study were an immunostaining score of 180/HPF for PD-L1 and 177/HPF for CTLA-4. However, as these values were determined by only 41 CMGTs, further prospective studies containing larger numbers of dogs are needed to assess the reliability of these identified cut-offs.

Blockade of immune checkpoint pathways using anti-PD-L1 and anti-CTLA-4 monoclonal antibodies (mAbs) is currently used to treat a variety of different human cancer types including non-small cell lung cancer, urothelial cancer, Merkel cell carcinoma, renal cell carcinoma, and triple-negative breast cancer.[12,40] In dogs, a rat–dog chimeric anti-PD-L1 mAb was found to be safe and well tolerated by dogs with oral malignant melanomas and undifferentiated sarcomas with a reported response rate of 14.3% (1/7) and 50% (1/2) respectively.[27] At present, surgical tumour excision is the most common treatment modality for CMGTs. However, it has been estimated that 25% of dogs with CMGTs cannot

be successfully treated with surgery alone, and these patients are considered as candidates for adjuvant therapy.[9,42] While additional studies are required, the results of the present study suggest that blockade of PD-L1 or CTLA-4 could potentially have some therapeutic benefits in dogs with CMGTs.

Immunostaining of PD-L1 was present on both neoplastic cells and immune cells that were infiltrating the tumour. The presence of PD-L1 on infiltrating immune cells has also been reported in human cancers. However, whether the presence of PD-L1 on tumour infiltrating cells helps predict subsequent tumour behaviour or response to PD-L1 blockade therapy is uncertain.[1,19,44] In the present study, neither the presence of PD-L1 nor CTLA-4 immunostaining in tumour-infiltrating cells was associated with the subsequent development of metastases, suggesting this feature is not prognostic for CMGTs.

Immunostaining for PD-L1 was observed in the nuclei of neoplastic mammary tumour cells in 11 of the 40 malignant mammary tumours included in the present study. Nuclear PD-L1 immunostaining was reported to be associated with more aggressive neoplasm behaviour in one study of human cancers[33], although other studies have reported nuclear PD-L1 immunostaining as an artefact.[29,35] The present study did not identify any association between nuclear PD-L1 immunostaining and disease outcome of CMGTs and it is uncertain whether the PD-L1 immunostaining observed was real or artefactual.

In the present study, both PD-L1 and CTLA-4 immunostaining was higher in malignant CMGTs than in benign neoplasms. This is in agreement with a previous study that also reported higher PD-L1 immunostaining in malignant CMGTs compared to benign CMGTs.[41] The higher PD-L1 and CTLA-4 immunostaining in malignant neoplasms supports the hypothesis that inhibition of an immune reaction due to these proteins results in a more aggressive neoplasm phenotype.

Tumour histological grade was independently prognostic of survival times of the dogs with malignant CMGTs in the present study. This was consistently observed in the prognostic studies included in Chapter 4 and Chapter 5 of this thesis. Similar prognostic significance of tumour histological grade for CMGTs has been previously reported by univariate[36] and multivariate analyses.[20,34] However, tumour size or presence of tumour emboli were not prognostic of survival times of dogs with malignant CMGTs in the present study. In contrast, several previous studies have shown that tumour size and presence of tumour emboli are prognostic of survival times of dogs with malignant CMGTs. [17,38,36] The possible causes for the discrepancy of results between studies are similar to what is described in section 4.4 of Chapter 4 of this thesis.

One limitation of the present study was the lack of tumour staging at diagnosis. This suggests it is possible that some CMGTs could have metastasised prior to the neoplasm being excised. Additionally, due to the nature of the study, it was impossible to definitively exclude the possibility that some of the dogs with non-metastatic CMGTs had developed clinically silent metastatic disease during the study. However, even if some of the non-metastatic CMGTs had developed clinically silent metastases, the fact that these neoplasms remained clinically silent suggests that metastasis either happened late in the study or these metastases that developed were less clinically aggressive. The clinical relevance of the findings of this study are supported by the significant differences in survival times between dogs with CMGTs with a high expression of the immune checkpoint proteins and dogs with low expression of these proteins.

Another limitation of the present study is the relatively small number of cases. However, significant differences in PD-L1 and CTLA-4 protein and gene expression were detected between metastatic and non-metastatic CMGTs suggesting the numbers included were adequate. Nevertheless, studies with larger numbers of cases are required to identify how PD-L1 and CTLA-4 protein and gene expression relate with other existing prognostic indicators including tumour stage and the histological-sub types of mammary neoplasms.

A final hierarchal multivariate analysis was conducted to see which of the cancer associated inflammation-related prognostic markers investigated in this thesis were independently prognostic of survival times of dogs with malignant mammary neoplasms. PD-L1 immunostaining and stromal mast cell density were independently prognostic, suggesting that these markers are capable of being used alone for prognostic determination of CMGTs. In contrast, other promising markers including CTLA-4 immunostaining, CXCL12 and CCL5 were not independently prognostic of survival times. In the case of CTLA-4, the findings in this chapter suggest that CTLA-4 immunostaining is prognostic; for example, the significant negative correlation between CTLA-4 immunostaining and survival times of dogs. However, CTLA-4 immunostaining lost its prognostic ability when tested with several conventional prognostic factors using the hieracheal multivariate analysis. This suggests that malignant mammary neoplasms which had higher CTLA-4 immunostaining scores developed metastasis not only because they had increased number of CTLA-4 molecules on tumour cells but also because those tumours were either large, high grade tumours or had histological evidence of lympho-vascular invasion. The same would also be true for the chemokines CXCL12 and CCL5. Therefore, more studies with large numbers of cases are necessary to investigate CTLA-4 immunostaining and chemokine expression in relation to conventional prognostic factors. This suggested that these two markers are capable to use alone for prognostic determination of CMGTs while other markers may be useful to complement the diagnosis provided by other conventional prognostic markers available for canine mammary neoplasms.

In conclusion, the study presented in this chapter is the first time that immune checkpoint proteins have been investigated in a series of malignant CMGTs of which the clinical outcome was known. The results revealed that increased production of these proteins was significantly associated with the development of metastatic disease and reduced survival times of affected dogs. As these two proteins influence the biological behaviour of CMGTs, they may be important as prognostic markers and as therapeutic targets for these common canine neoplasms.

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Chapter 7 : Final Discussion

Tumour-associated inflammation has recently gained widespread attention due to the interesting association observed between inflammation and biological behaviour of cancers.[1,14] Numerous studies have demonstrated that certain components of tumour-associated inflammation (TAI) are useful prognostic markers and therapeutic targets for many types of cancer in humans.[14,1] Breast cancer is one of the most common neoplasms among women worldwide. Current research on breast cancer is mainly focused on identifying novel prognostic markers to accurately predict the disease outcome of patients and developing new therapeutics to treat the disease more effectively. During the past two decades, the utility of TAI related components as prognostic markers and therapeutic targets in breast cancers has been extensively researched. This thesis was inspired by the novel and interesting findings of TAI in human breast cancers and the clinical and molecular similarities between mammary neoplasms in humans and dogs.[13]

The main aim of this thesis was to identify whether a group of TAI related prognostic markers adopted from human breast cancer studies are similarly prognostic in canine mammary gland tumours (CMGTs). As hypothesised, most of the tested prognostic markers were prognostic in CMGTs as they are in human breast cancers. Stromal mast cell density was the most promising prognostic marker due to both its prognostic value and the relative ease of implementing the technique in a diagnostic laboratory. The other markers, including chemokines and immune checkpoint molecules, carry the potential to be adopted for routine diagnostics but need further research to make them more user-friendly and cost-effective prognostic markers. This chapter will discuss the feasibility of these prognostic markers for routine prognostic determination in a diagnostic laboratory. Further, this discussion will include possible ways to further improve the prognosis provided by these prognostic markers. Additionally, this chapter will discuss some of the

challenges associated with retrospective prognostic studies and a few other interesting findings from two surveys carried out for data collection.

Of the prognostic markers identified by this thesis, stromal mast cell density appeared to be the most appropriate marker for routine prognostic determination in a diagnostic laboratory due to several reasons. Firstly, veterinary pathologists rarely use additional laboratory diagnostics other than HE staining for prognostic determination in CMGTs mostly due to cost. For instance, many potential molecular prognostic marker assays for CMGTs use immunohistochemistry (IHC) or PCR techniques which require expensive reagents. Conversely, the reagents used for toluidine blue staining are relatively inexpensive and therefore it is more economical than IHC or PCR. Secondly, sample processing protocols and preferred brands of antibodies or primers used for IHC or PCR may vary between diagnostic laboratories. Currently there is no consensus over how to manage inter-laboratory variability of many IHC or PCR-based prognostic marker assays developed for dogs. In contrast, toluidine blue staining is already routinely performed by veterinary diagnostic laboratories and therefore many laboratories follow a standard protocol.[11,6] Considering these advantages, it is likely that veterinary pathologists would be more likely to use stromal mast cell density assessed by toluidine blue staining to complement the prognosis provided by HE staining for CMGTs.

One potential disadvantage of measuring stromal mast cell density is the requirement to manually count mast cells, which is a time-consuming process, and cell counts may vary between observers. Only a single observer performed the mast cell counting for the CMGTs included in this thesis and therefore the inter-observer variability of mast cell counting could not be evaluated. Recently, there is growing interest in the scientific community to use computer-based software programs to automate cell counting in histological sections. These software programs have been shown to make cell counting in histological sections easy and rapid while minimising inter-observer and inter-laboratory variability. Therefore, it would be useful to further investigate methods to automate mast

cell counting in histological sections of CMGTs before adopting measuring stromal mast cell density as a routine diagnostic procedure.

In addition to stromal mast cell density, this thesis identified that increased gene expression of chemokines CCL5, CXCL12 and chemokine receptors CXCR4 and CCR9 was associated with a poor disease outcome in dogs with malignant CMGTs. Similar human breast cancer studies have also reported alike associations and discussed the possibility of using these chemokines and chemokine receptors as prognostic markers or therapeutic targets for breast cancers. These previous studies have shown that prognostic significance and therapeutic effects occur consequent to downstream signalling pathways activated by chemokines and chemokine receptors.[8] The activated downstream signalling pathways, including phosphoinositide 3-kinase and mitogen activated protein kinase pathways, are involved in cell proliferation, motility, and expression of matrix metalloproteinases.[8] Neoplasms with aggressive behaviours have higher cell proliferation rates while synthesis of matrix metalloproteinases has found to be altered in these neoplasms making neoplastic cells more prone to get dislodged from the primary sites. However, some of these human breast cancer studies have suggested that *in vivo* functions of these chemokines and chemokine receptors may be more complex than what is predicted by *in vitro* studies due to the complexity of chemokine signalling in the tumour microenvironment (TME). For example, the ability of multiple chemokines to bind with the same receptor and the ability of a single chemokine to bind with multiple receptors may create redundant signalling and thereby interfere with prognostic efficacy or therapeutic potential of a particular chemokine or chemokine receptor. [8] Therefore, it may be useful to investigate the downstream signalling pathways activated by the chemokines and chemokine receptors shown to be prognostic in CMGTs in this thesis using *in vivo* models to better clarify their prognostic or therapeutic roles.

Many chemokines have been reported in the tumour microenvironment and there are complex interactions between them. Considering these complexities, human breast cancer studies have suggested that using a single chemokine or a chemokine receptor as a

prognostic marker or therapeutic target may have limitations. To overcome these limitations, some recent human breast cancer studies have used combinations of chemokines to predict cancer recurrence and metastasis.[17,9] Interestingly, these multi-targeted approaches were more promising than single chemokine or chemokine receptor-targeted approaches.[17] There have been attempts to develop some of these chemokine combinations into commercial diagnostic kits for routine laboratory use. The initial development and production cost of such diagnostic kits is high. However, considering the number of breast cancer patients seeking precise prognostic determination and alternative therapies, the unit price of a kit would become affordable over time. Further research is necessary to integrate the canine chemokines and chemokine receptors identified in this thesis into a clinically applicable diagnostic algorithm in dogs. However, compared to human breast cancer patients, relatively lower numbers of canine patients with mammary tumours seek sophisticated diagnostics or adjuvant therapies. Therefore, although it may be possible to develop similar types of diagnostic tools for canine patients with mammary tumours, the commercial success of such products in the veterinary field is currently uncertain.

The findings in Chapter 6 of this thesis suggested that immune checkpoint molecules: PD-L1 and CTLA-4, are potential prognostic markers for determining the biological behaviour of CMGTs. These two immune checkpoints have been identified as prognostic markers for many different types of human cancers.[2] Consequent to the success of immune checkpoint inhibition as a novel cancer treatment modality, assaying for immune checkpoints has become not only a useful diagnostic tool for prognostic determination but, also a prerequisite for immune checkpoint inhibition therapy. For example, immunostaining for PD-L1 is an FDA-approved diagnostic test and a prerequisite for treatment with anti-PD-1 / PD-L1 antibodies.[2] Therefore, human oncologists often request these immunoassays from diagnostic laboratories. Prognostic determination of canine cancers using immune checkpoint assays is still a novelty in the field of veterinary oncology. In addition, immune checkpoint molecules have been researched only in a limited number of canine cancers. Thus, although PD-L1 and CTLA-4 show considerable promise as both prognostic markers and potential therapeutic targets, these could not be readily adopted for prognostic

determination in a diagnostic laboratory or treating dogs with mammary neoplasms. Therefore, it is necessary to have further investigations to identify the availability and prognostic significance of PD-L1 and CTLA-4 immune checkpoint molecules in other canine neoplasms which may increase the awareness of the utility of these markers creating a demand for testing them.

Commercially available antibodies produced against human immune checkpoint proteins (PD-L1 and CTLA-4) were used for immunostaining of canine tumour tissues in this thesis. Currently, no canine-specific anti-PD-L1 or anti-CTLA-4 antibodies are commercially available. Even though canine-specific antibodies may be available in future, they are unlikely to be cheaper than antibodies developed against the corresponding human proteins. Therefore, the ability to use antibodies developed against human immune checkpoint proteins to detect the corresponding canine proteins shown in this thesis may facilitate developing these immunoassays into diagnostic assays affordable to dog owners. Despite these promising attributes, developing PD-L1 or CTLA-4 immunostaining into routine diagnostic assays would have other obstacles. For example, the average cost of PD-L1 immunostaining available for human patients is around US$500.[10] Although the human diagnostic laboratories advertise the cost for a single test, samples are mostly processed in batches using commercial reagent kits.[10] This is feasible for human laboratories as immune checkpoint assays are frequently requested.[2] In contrast, it is less likely for veterinary diagnostic laboratories to receive canine cancer biopsies at a similar rate to allow batch processing. If a laboratory has to perform immunostaining for samples individually as they arrive this will result in a much higher cost. This suggests that veterinary diagnostic laboratories may be less likely to invest in developing an immune checkpoint assay for routine testing canine neoplasms. However, there are ongoing research projects which have shown promising preliminary findings on using immune checkpoint inhibition to treat canine neoplasms. If these investigations are able to prove that immune checkpoint inhibition is a successful method to treat canine neoplasms then dog owners may be more willing to pay the cost for immune checkpoint testing, making diagnostic laboratories more willing to invest in commercialising immune checkpoint assays.

The samples used for the prognostic marker assays described in this thesis were surgically excised, formalin-fixed canine mammary tumour tissues. However, formalin fixation is time consuming and therefore does not facilitate rapid testing. In human medicine, diagnostic assays are available to determine chemokines or immune checkpoint molecules in blood or serum of cancer patients.[10] These assays are useful as sample collection is relatively non-invasive and quicker than using surgically excised, formalin-fixed neoplasms. Most importantly, these assays also provide sufficiently accurate prognostic information to be useful for decision making in patients with breast cancers. Currently, no such diagnostic applications are available to determine chemokines or immune checkpoint proteins in blood or serum of dogs with CMGTs. However, some past studies have shown that chemokines or soluble isoforms of immune checkpoint proteins are present in blood and serum of dogs similar to humans.[16,5] Thus, it would be useful to investigate bioavailability of chemokines and immune checkpoint proteins in blood or serum of dogs with CMGTs to determine whether the concentrations of these proteins differ significantly between dogs with better or worse disease outcomes.

While identifying prognostic significance of TAI related prognostic markers, this thesis highlights certain important aspects of choosing retrospective study models for canine prognostic marker studies. Retrospective studies are less time consuming and relatively inexpensive compared to prospective studies. However, a big disadvantage of using a retrospective study in this thesis was the inability to obtain necessary data for mammary tumour staging in most cases. Pre-surgical tumour staging is important to determine the extent of tumour metastasis at the time of surgical excision of a mammary tumour. Inability to obtain tumour staging has not been reported in similar types of human breast cancer studies. The discrepancy is likely due to the higher standard of care in human patients compared to canine patients with mammary neoplasms. Cancer staging is performed routinely for human breast cancer patients due to its importance in determining therapeutic options for individual patients. However, the online survey conducted for data collection in this thesis revealed that tumour staging is not routinely performed in most canine patients with mammary neoplasms. According to the correspondence had with the submitting veterinarians, the main reason for not staging

mammary neoplasms was the high cost associated with the necessary diagnostic tests. The lack of staging data was a big disadvantage of the retrospective study in this thesis as it is not possible to go back and stage a neoplasm. Pre-surgical staging of a neoplasm requires thoracic and abdominal radiographs and fine needle aspirations or core biopsies of regional lymph nodes. However, in a retrospective study, as the surgical excision has already been performed it is impossible to repeat radiographs or obtain lymph node biopsies, making tumour staging not possible unless the radiographs and biopsies have been previously collected. In contrast, staging could be mandated in a prospective study of these neoplasms. Another difficulty encountered with retrospective studies is obtaining follow-up data of the patients. In this thesis, many mammary neoplasm cases which fulfilled basic inclusion criteria identified from the IDEXX database on primary search had to be excluded due to the unavailability of follow-up data. In the majority of cases, the patients had been lost for follow-up due to change in the veterinary care provider. Additionally, follow-up data of some dogs were incomplete as the clinical records of some dogs which had died by the time of the survey had been removed from veterinary clinic databases due to technical reasons.

Many human breast cancer studies that correlated the expression of TAI related prognostic markers with tumour metastasis measured the expression of the particular markers not only in primary tumours but also in metastatic sites.[12,15] This approach has been helpful to identify how some of these prognostic markers influence tumour metastasis to specific sites. However, due to the retrospective nature of the data collection process included in this thesis, it was not possible to obtain biopsies or post-mortem samples of metastases from most of the cases that were reported to have clinical evidence of tumour metastasis. In fact, post-mortem examinations had not been performed on most of the dogs, as per the wishes of the dog owners, while in the cases where post-mortem examinations were performed, not all metastatic sites were sampled and submitted for histopathology due to economic reasons. The inability to compare marker levels between primary tumours and metastatic sites was a limitation of the studies reported in this thesis.

The two surveys included in this thesis were carried out to collect samples for the prognostic marker studies. The New Zealand survey included 896 cases, of which 53 cases were used for prognostic marker studies as follow-up information was able to be retrieved successfully. The Sri Lankan survey included 72 cases, but unfortunately there was insufficient data on disease outcome to use these cases for prognostic marker evaluation. However, both surveys provided a large number of CMGT cases which were helpful to determine the clinicopathological features of mammary neoplasms in dogs in Sri Lanka and New Zealand. This was important as no previous studies have investigated mammary neoplasia in dogs in either country. Further, analysis and comparison of the clinicopathological data of mammary neoplasms in dogs in Sri Lanka and New Zealand provided some interesting insights which may pave the way for future studies to better understand mammary gland diseases in dogs in these two countries.

The most noteworthy feature of the two surveys was the marked difference in proportions of histologically-malignant neoplasms in dogs between the two countries. In Sri Lanka 88% of dogs had malignant mammary neoplasms while malignant neoplasms were found only in 55% of dogs in New Zealand. The reason for the observed difference is unknown. It is possible that dogs in Sri Lanka are more frequently exposed to carcinogens which promote development of malignant neoplasms than dogs in New Zealand. Possible examples might include exposure to pesticides and the use of steroid contraceptives to prevent unwanted pregnancies in female dogs in Sri Lanka. Alternatively, the discrepancy could be due to differences in veterinary care received by dogs in these two countries. It is possible that dogs in New Zealand are more frequently seen by veterinarians than dogs in Sri Lanka, facilitating early detection of benign mammary neoplasms which are generally small and only identified on careful examination. Additionally, the two veterinary hospitals selected for the prospective survey in Sri Lanka were referral centres. Therefore, these practices may have received CMGT cases referred by the regional veterinarians which are more likely to be large malignant tumours than small benign tumours. Therefore, future studies designed to investigate the factors responsible for the differing rates of malignancy would be helpful to better understand the disease process in general as well as to identify contributory factors which may have regional importance.

The statistical analyses of surveys from both New Zealand and Sri Lanka identified that tumour size is useful to predict whether a mammary neoplasm on a dog is histologically malignant or benign, with larger neoplasms more likely to be malignant. However, there was no statistically significant difference in size between non-neoplastic mammary lesions and neoplastic mammary lesions. Therefore, a clinician would not be able to use size as a criterion to differentiate between neoplastic and non-neoplastic lesion, so the importance of size in differentiating between histologically-benign or malignant neoplasms is not useful in a clinic setting. Given these limitations, this thesis highlights the importance of using additional diagnostics such as cytology and histopathology to diagnose mammary gland diseases in dogs shown by previous studies.

Mammary neoplasms were common in intact female dogs in both Sri Lanka and New Zealand although the percentage of intact dogs from Sri Lanka was higher than New Zealand. This was not unexpected as routine dog spaying is practiced more frequently in New Zealand compared to Sri Lanka. Interestingly, the Sri Lankan survey identified that nearly half of the intact dogs included in the survey were nulliparous. The parity of the intact New Zealand dogs was unknown as it was not recorded for the cases submitted to IDEXX Laboratories, New Zealand. The hormonal aetiology of canine mammary neoplasms is well known and the higher percentage of affected dogs being intact is therefore justifiable. Nulliparity may cause prolonged exposure of mammary neoplasms to reproductive hormones elevating the risk of developing mammary neoplasms compared to uniparous or multiparous dogs. However, this cannot be confirmed without the knowledge of proportion of nulliparous dogs in the Sri Lankan female dog population. Therefore, in a future study it would be interesting to identify whether being nulliparous is a risk factor for mammary gland neoplasia in dogs.

Multiple mammary tumours were reported in 20% and 12% of dogs in Sri Lanka and New Zealand respectively. These proportions were markedly lower than the 60–70% of cases with multiple mammary neoplasms reported in previous studies.[7] It is unknown why the proportions of dogs with multiple mammary neoplasms in New Zealand and Sri Lanka are

relatively lower than some previous reports. It has been suggested that multiple mammary neoplasms develop subsequent to simultaneous exposure of multiple mammary glands to reproductive hormones.[3,18] Therefore, the reason for the relatively low proportion of multiple neoplasm cases in New Zealand could be attributed to routine early spaying of female dogs which prevents the prolonged exposure of mammary gland tissues to reproductive hormones. Alternatively, being a laboratory-based survey, the NZ survey may not have included all the CMGT diagnosed, but the cases of which the samples had been submitted for histopathology, artificially reducing the multiple CMGT cases. However, this does not similarly apply to the Sri Lankan dogs as in Sri Lanka the majority of dogs are either intact or spayed late in their reproductive life. Therefore, it is likely that there are other factors which may contribute to lower percentage of dogs with multiple mammary neoplasms in Sri Lanka and NZ and future studies are necessary to identify these factors.

In conclusion, this thesis identified that several TAI related markers shown to be prognostic in human breast cancers are similarly prognostic in canine mammary neoplasms. Of these prognostic markers, stromal mast cell density was the most useful and could be easily adopted for prognostic determination of CMGTs in a veterinary diagnostic laboratory. The other prognostic markers, including chemokine and chemokine receptors and immune checkpoint molecules, were shown to be useful in prognostic determination in malignant canine mammary neoplasms. However, these assays would be more difficult and expensive to implement in a veterinary diagnostic laboratory compared to the method for measuring stromal mast cell density. Therefore, further studies are necessary to identify ways to make these chemokine and immune checkpoint molecule prognostic marker assays more user-friendly and cost effective. Overall, the investigations carried out in this thesis are helpful to broaden our understanding of how cancer-associated inflammation influences the behaviour of canine cancers while paving the way for further studies to develop these markers for use in routine laboratory diagnostics. The two surveys carried out for data collection helped to identify the clinicopathological features of mammary neoplasms in Sri Lanka and New Zealand which have not been studied previously. The findings of these two surveys may serve as a platform for future studies on canine mammary neoplasms in these countries, particularly investigating the regional factors that influence the development of

malignant mammary neoplasia in dogs. Additionally, the statistical analyses carried out in this thesis helped to confirm the limitations of some of the currently used patient and tumour-related conventional prognostic markers identified by previous studies.

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Appendix A: List of publications and statement of contribution

Publications

Ariyarathna H, De Silva N, Aberdein D, et al. Clinicopathological diversity of canine mammary gland tumors in Sri Lanka: A one-year survey on cases presented to two veterinary practices. *Vet Sci*. 2018;5: 46.

Ariyarathna H, Thomson N, Aberdein D, Munday JS. Low stromal mast cell density in canine mammary gland tumours predicts a poor prognosis. *J Comp Patholol*. 2020; 1:29-38.

Ariyarathna H, Thomson N, Aberdein D, Munday JS. Chemokine gene expression influences metastasis and survival time of female dogs with mammary carcinoma. *Vet Immunol Immunopathol.* 2020; 13:110075.

Ariyarathna H, Thomson NA, Aberdein D, Perrott MR, Munday JS. Increased programmed death ligand (PD-L1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) expression is associated with metastasis and poor prognosis in malignant canine mammary gland tumours. *Vet Immunol Immunopathol*. 2020; 20:110142.

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We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Harsha Subhani Ariyarathna
Name/title of Primary Supervisor:	Professor. John S Munday
Name of Research Output and full reference:	
Ariyarathna H, De Silva N, Aberdein D, et al. Clinicopathological diversity of canine mammary gland tumors in Sri Lanka: A one-year survey on cases presented to two veterinary practices. Vet Sci. 2018;5: 46.	
In which Chapter is the Manuscript /Published work:	2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	70%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate designed the survey, and prepared the manuscript. The co-authors provided the technical supppprt and helped with the preperation of the manuscript.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Harsha Ariyarathna Digitally signed by Harsha Ariyarathna Date: 2020.11.03 06:59:42 +05'30'
Date:	2020-11-3
Primary Supervisor's Signature:	John Munday Digitally signed by John Munday Date: 2020.11.04 11:09:35 +13'00'
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Name of candidate:	Harsha Subhani Ariyarathna
Name/title of Primary Supervisor:	Professor. John S Munday
Name of Research Output and full reference:	
Mammary gland disease in dogs in New Zealand: a review of 896 canine mammary gland samples submitted to diagnostic laboratories in New Zealand	
In which Chapter is the Manuscript /Published work:	3
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	60%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate performed the database survey, data analysis and prepared the manuscript. The co-authors helped with designing the survey and helped with the preperation of the manuscript.	
For manuscripts intended for publication please indicate target journal:	
New Zealand Veterinarry Journal	
Candidate's Signature:	Harsha Ariyarathna Digitally signed by Harsha Ariyarathna Date: 2020.11.03 06:59:42 +05'30'
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In which Chapter is the Manuscript /Published work:	4
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	70%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate designed and carriedout the experiments, and prepared the manuscript. The co-authors provided technical support and helped with the preperation of the manuscript.	
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Date:	2020-11-03
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Name/title of Primary Supervisor:	Professor. John S Munday
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Ariyarathna H, Thomson N, Aberdein D, Munday JS. Chemokine gene expression influences metastasis and survival time of female dogs with mammary carcinoma. Vet Immunol Immunopathol. 2020; 13:110075.	
In which Chapter is the Manuscript /Published work:	5
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	70%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate designed and carriedout the experiments, and prepared the manuscript. The co-authors provided technical support and helped with the preperation of the manuscript.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Harsha Ariyarathna Digitally signed by Harsha Ariyarathna Date: 2020.11.03 06:59:42 +05'30'
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In which Chapter is the Manuscript /Published work:	6
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	70
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate designed and carriedout the experiments, and prepared the manuscript. The co-authors provided technical support and helped with the preperation of the manuscript.	
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