Understanding the prognosis and progression of soft tissue sarcoma in the dog

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Jonathan Peter Bray

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Abstract

Soft tissue sarcoma (STS) are tumours derived from tissues of mesenchymal origin. Local recurrence of the tumour following surgical resection is the characteristic challenge in the management of STS. There are currently no prognostic tests that can reliably predict which tumours have a higher or lower risk of recurrence. The aim of the studies contained in this thesis was to investigate aspects of STS biology to identify new prognostic markers. A large archive of STS was established with patient outcomes determined by questionnaire. Tissue was subsequently analysed using immunohistochemistry and reverse transcriptase-polymerase chain reaction to understand the role of two molecules – vascular endothelial growth factor (VEGF) and decorin – in influencing tumour behaviour. This study revealed that when the tissue stroma surrounding the tumour cells had a strong immunostaining intensity for decorin, the risk of tumour-related death was significantly reduced. In addition, STS with a high immunostaining for VEGF were more than 7 times more likely to recur, and 5 times more likely to cause the death of the dog. When the immunostaining characteristics for VEGF and decorin were combined with other patient and tumour features into a predictive algorithm called a nomogram, it was possible to determine, with almost 100% accuracy, which dogs would remain disease-free 3 years after surgery. The importance of VEGF in the progression of tumour growth was

subsequently demonstrated by treating dogs with haemangiosarcoma (HSA) – a mesenchymal tumour with many characteristics similar to STS – with thalidomide. Thalidomide is a potent antagonist of VEGF, but also has a number of other modulating influences on the tumour microenvironment. Dogs treated with thalidomide survived significantly longer than dogs that did not receive this drug, suggesting that thalidomide can slow the ability for residual microscopic tumour cells to develop into a grossly visible, and life-threatening tumour. An analysis of metastatic lesions that developed in dogs treated with thalidomide revealed that immunostaining for VEGF was significantly reduced. This suggests that thalidomide may be a useful adjuvant therapy for dogs with STS that are considered to be at high risk of recurrence after surgery, as determined by their VEGF immunostaining intensity.

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Dedication

This thesis is dedicated to my parents, Margaret & Ken. They were two incredibly kind, generous and supportive people, who loved each other and their extended family with all of their heart. I am sad not to have been able to finish this thesis while they were still alive. I know they would have been more proud than I am.

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Abbreviations

AgNOR	Argyrophilic nucleolar organizer region
AUC	Area under the curve
BLASTn	Basic local alignment search tool
C-index	Concordance index
CDK-1	Cyclin-dependent kinase **1**
CI	Confidence interval
CNS	Central nervous system
CT	Computed tomography
Ct	Cycle threshold
DAB	Diaminobenzidine
DCN	Decorin
DFI	Disease-free interval
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DV200	Fragment analysis metric
Dxy value	Somer's D
ECM	Extracellular matrix
EGF	Epidermal growth factor
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FNA	Fine needle aspiration

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIST	Gastrointestinal stromal tumour
Gy	Gray
HGF	Hepatocyte growth factor
HIF-1a	Hypoxia inducible factor
hpf	High power fields
HR	Hazard ratio
HSA	Haemangiosarcoma
HTFM	Histological tumour free margin
IHC	Immunohistochemistry
IL-1β	Interleukin 1β
IL-6	Interleukin 6
LOX	Lysyl oxidase
MI	Mitotic index
MMP-9	Matrix metallopeptidase 9
mRNA	Messenger RNA
MST	Median survival times
MTC	Metronomic chemotherapy
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells, a type of cytotoxic lymphocyte
NSAID	Non-steroidal anti-inflammatory drug
NZVP	New Zealand Veterinary Pathology
OR	Odds ratio
PCR	Polymerase chain reaction

PDGF	Platelet derived growth factor
PERM	Paraffin-embedded RNA metric
PNST	Peripheral nerve sheath tumour
PWT	Perivascular wall tumour
qPCR	Quantitative PCR
RIN	RNA integrity number
ROC	Receiver operating characteristic
RT	Radiotherapy
RT-PCR	Reverse transcriptase polymerase chain reaction
STEPS	System for Thalidomide Education and Prescribing Safety
STS	Soft tissue sarcoma
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-alpha
TNM system	Tumour, node, metastasis
Treg	Regulatory T cells
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor
VEGFR-3	VEGF receptor-3

CHAPTER 1

Prologue

1.1 Introduction and Problem Statement

Soft tissue sarcoma (STS) are tumours derived from tissues of mesenchymal origin.[1-4] They are remarkably common, representing between 9 and 15% of all cutaneous or subcutaneous tumours.[3, 5, 6] These neoplasms are therefore familiar to most veterinarians.

Animals with a STS usually present with a subcutaneous mass that can range in size from <1cm to more than 10 cm.[3, 6] Because of their size and/or anatomical location, STS can provide the clinician with significant challenges with respect to surgical management. However, with adequate treatment, many dogs with STS can experience prolonged survival with mean survival times ranging from 1013 to 1796 days (3-5 years).[7, 8]

Despite these favourable figures, currently almost one in five patients with a STS will die due to the neoplasm. Local recurrence is the most common event following surgery with estimates of recurrence rates ranging from 7 to 75% of patients.[1, 7-10]. Local tumour recurrence is consistently associated with reduced overall survival and tumour-related death; in one study, tumour recurrence was associated with a more than 5-fold increased risk of death (Hazard Ratio (HR) 5.2; P<0.0001; 95% Confidence interval (CI): 3.1–9.0);

other investigators have reported similar findings.[1, 8, 11] There is currently insufficient evidence to determine whether adjuvant treatment with chemotherapy or radiotherapy can prevent or slow local or distant recurrence of a STS following surgery.[6, 12, 13] Development of additional adjuvant treatments to help manage STS is required.

The ultimate goal of cancer surgery is to prevent local recurrence of the tumour. To ensure complete removal, standard recommendations for surgical margins around a STS have prescribed wide excision of the tumour, obtaining 3cm of normal tissue about the entire circumference of the tumour and one clean fascial plane deep to the tumour.[2, 3] These recommendations were established because an early clinical study on STS demonstrated high rates of local recurrence and short disease-free intervals when the tumour was removed without planned resection margins.[1] However, some studies have suggested that the extent of resection does not influence the disease-free interval or overall survival.[7, 14] Interpretation of current evidence suggests that no single width of surgical margin will provide effective treatment of every STS;[15, 16] it is possible that some tumours could be successfully managed with excision margins of just a few millimetres, while others require more extensive resections. However, there are currently no diagnostic tests that can reliably predict the amount of surgical margin required for an individual tumour.

Surgeons currently rely on analysis of the resected tissue to determine whether a surgery has been successful and to establish whether the dog may be at risk of recurrence. This analysis includes determination of the tumour

grade, mitotic rate and whether the resection margins are clear of tumour cells.[5] With STS, higher rates of recurrence are reported with more aggressive, high grade tumours and/or when surgical margins about the tumour have been inadequate.[2, 5, 7-9, 11] However, many of the existing techniques used to analyse the resected tissue have limitations which can affect their accuracy.[5] Overall, this inaccuracy means that a STS will recur in almost 1 in 10 dogs despite post-operative analysis suggesting it was completely resected.[8, 9, 11, 17-22] Conversely, even when histology has identified tumour cells at the edge of the resected tissue margin, local recurrence does not occur in more than 70% of these dogs. These inconsistencies can have an adverse impact on the clinical management of a dog with a STS. The development of alternative prognostic markers that provide better correlation with actual patient outcomes are required.

1.2 Research Aim

The aim of this study is to investigate previously unexamined cell function pathways that may be important in determining STS biology using immunohistochemistry (IHC) and polymerase chain reaction (PCR) methods. By identifying new biomarkers that may be associated with increased or decreased rates of tumour recurrence after surgical excision, it is hoped these may provide a more accurate prognosis; the biomarkers could then be used to provide improved guidance on optimal tumour control. In addition, by examining molecular control pathways in STS it may be possible to identify opportunities for new adjuvant treatments to improve patient outcomes.

1.3 Overview of Thesis Structure

Chapter 2 reviews the current knowledge and treatment guidelines for canine STS, and makes some comparisons with the equivalent human disease. Attention is given to the current limitations of determining surgical margins and tumour prognosis. A review of evidence that shows how the persistence of tumour cells after surgery, which ultimately leads to tumour recurrence, could lend support for the identification of relevant biomarkers.

To support the laboratory analyses required for biomarker discovery, an archive of clinical material was required. A retrospective study was performed to determine the outcomes for 350 dogs treated with STS in first opinion practice. This is the largest study ever performed for this tumour type in the dog; the details of which are outlined in Chapter 3.

In Chapter 4, the results of an immunohistochemistry study to examine the role of two possible prognostic markers, vascular endothelial growth factor (VEGF) and decorin, are described. These molecules are known to play important roles in the tumour microenvironment, with particular influence on angiogenesis and tumour cell migration. Chapter 5 details the results of a study using reverse-transcriptase polymerase chain reaction that was designed to validate and further characterise the expression of VEGF within the tumour tissues.

Combining the discoveries of these proceeding studies, Chapter 6 describes the development and validation of a predictive algorithm that could allow a clinician to determine the likelihood that a particular STS will recur following

surgery. This algorithm uses characteristics of STS biology obtained from clinical examination and histologic analysis of the tumour to provide a prediction on whether or not a particular tumour is likely to recur after surgery.

In Chapter 7, a prospective clinical study to investigate whether thalidomide can prolong the survival time of dogs with haemangiosarcoma (HSA) is described. Thalidomide is known to be a potent antagonist of VEGF. Haemangiosarcoma is a mesenchymal tumour with some cellular and molecular characteristics similar to STS.[23-25] This tumour type was chosen as a proxy for STS as it has a rapid disease progression and almost 100% incidence of metastatic disease. A similar study using STS would have taken several years and required hundreds of cases, due to the slow progression of this tumour and relatively low incidence of metastases.

Finally, in Chapter 8, the VEGF immunostaining in HSA of dogs that received thalidomide was compared to the VEGF immunostaining in HSA of dogs that did not receive this drug.

In Chapter 9, overall conclusions about the potential for the biomarkers described in this study will be discussed, and how they could be used to predict STS biology or to influence treatment decisions. The limitations of the research and possible direction of future studies will also be examined.

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Chapter 2

Background and a review of the literature

2.1 Introduction

Soft tissue sarcomas (STS) are a heterogeneous group of mesenchymal tumours. Soft tissue sarcomas may arise anywhere in the body but develop most commonly on the appendicular skeleton. They typically present as a firm, discrete and expansile mass. When treated appropriately, the prognosis for the majority of dogs is good, provided that complete removal of the tumour has been achieved. However, more than 20% of dogs will ultimately die from their STS either because they develop a recurrence of their tumour that is not resectable or they develop distant metastases.

Although they are common in dogs, many uncertainties surround the best options for clinical management of STS. The first part of this review provides an update on the current understanding of the diagnosis and management of canine STS. In the second part of this review, an in-depth analysis of the issues known to influence the prognosis of canine STS is provided.

2.2 Soft tissue sarcoma

Soft tissue sarcoma (STS) are tumours derived from mesenchymal tissues.[1-4] This derivation means they can arise at virtually any anatomical site. They

are common in the dog and represent approximately 15% of all skin and subcutaneous neoplasms.[3] Annual incidence rates of STS have been estimated to be between 35 and 142 tumours per 100,000 dogs at risk;[5] however, this data was derived almost 50 years ago from a veterinary cancer register that collated health data from a single county in California, USA. The reliability of this broad estimate for dogs living today is therefore difficult to determine. A more recent study from the United Kingdom (UK) evaluated the incidence of tumours using information from insurance records; this study revealed a similar incidence of 122/100,000 (95% CI 103-141) dogs/year.[6] In that study, soft tissue sarcoma was one of the most common malignant tumours to occur in the dog, second only to mast cell tumours. Again, this is a selected sample group as it has been estimated that only about 30% of dogs are covered by pet insurance in the UK.[7, 8] It is likely these owners are more motivated to seek veterinary investigation for various ailments, including palpable masses.

While STS tend to grow slowly and are considered to have a low metastatic potential, local recurrence of the tumour following surgical resection is common and has been reported to occur in up to 75% of patients.[9] Local recurrence is the most frequent reason for treatment failure in the management of STS and is consistently associated with reduced patient survival.[1, 3, 5, 10-12]

2.3 Nomenclature of soft tissue sarcoma

Soft tissue sarcoma develop from cells of mesenchymal origin, which includes muscle, neurovascular, connective and fatty tissues.[5, 13] Although the majority of masses will be subcutaneous or musculoskeletal in location, STS can also develop within cavity organs such as the lung, heart, liver, spleen, urogenital tract or gastrointestinal system, as well as retroperitoneal and mediastinal spaces.

The term "soft tissue sarcoma" is used to describe a group of mesenchymal tumour types that all have similar biological behaviours.[5] The features that characterise a STS are outlined in Table 2.1. Despite being a heterogenous group of tumours, they have been grouped together because it can be difficult to distinguish different subtypes by light microscopy alone.[13] Their biological behaviour is also considered fairly similar, so treatment recommendations are proposed as if all tumours in the STS group respond similarly.

There are several mesenchymal tumours that arise from soft tissue that are not considered to be STS.[13] This is because these tumours can usually be reliably identified on light microscopy or because their individual biological behaviour has a more defined character.[13] These mesenchymal tumours include: haemangiosarcoma, synovial cell sarcoma, gastrointestinal stromal tumours (GISTs), fibrosarcoma involving the oral cavity, and peripheral nerve sheath tumours that arise from the brachial or lumbosacral plexus. Some review papers have included rhabdomyosarcoma, lymphangiosarcoma and leiomyosarcoma to be part of the STS group,[3, 5] but most pathologists

generally consider these tumour types to be readily identifiable as individual tumours.[13]

Table 2.1:
Biological features that are common to soft tissue sarcoma (with modification from the original description by Withrow and MacEwan)[5]

- *an ability to arise from any anatomical site in the body*
- *appear as distinct, encapsulated tumours but on microscopic evaluation they have poorly defined margins and will infiltrate along tissue planes*
- *local recurrence is common after conservative excision*
- *metastasis occurs through a haematogenous route. Lymph node metastasis is uncommon.*
- *grossly detectable disease has a poor response to chemotherapy and radiation therapy*

The tumours that are typically included in the STS group arise from fibrous connective tissues, nervous tissues, adipose tissues, smooth muscle, skeletal muscle, and synovial tissues. They include fibrosarcoma, peripheral nerve sheath tumours (previously called neurofibrosarcoma or schwannoma), perivascular wall tumours (previously called haemangiopericytoma), liposarcoma, malignant fibrous histiocytoma, mesenchymoma, myxosarcoma, and undifferentiated sarcoma.[5, 9, 14] The term "spindle cell tumour" may also be used by some pathologists when a more precise identity of the STS subtype is not possible.[14] A summary of each of these tumour subtypes is detailed in Table 2.2.

Table 2.2:
Types of STS, as defined by their cell of origin and histological features that may be seen on light microscopy (modified from Dennis et al)[13]

Histological name	Previous names	Cell of origin	Histological features
Fibrosarcoma		Fibrous tissue	Interwoven bundles, herringbone pattern
Pleiomorphic sarcoma	Malignant fibrous histiocytoma	Fibrous tissue	Mix of fibroblastic and histiocytoid cells in storiform patterns, with a variable inflammatory infiltrate
Myxosarcoma		Fibrous tissue	Spindle-cells within a mucinous stroma
Peripheral nerve sheath tumours	Schwannoma Neurofibrosarcoma	Schwann cell Neurofibroblast	Interwoven bundles, whorls
Perivascular wall tumours	Haemangiopericytoma	Pericyte	Vascular growth pattern, perivascular whirling
Mesenchymoma		Multiple cell types	Multiple mesenchymal cell types, including osteoid, chondroid or collagenous matrix
Liposarcoma		Lipoblast	Polygonal cells with vacuolated cytoplasm
Rhabdomyosarcoma		Skeletal myocyte	Cytoplasmic striation, racket and strap cells
Lymphangiosarcoma		Lymph tissue	Irregular, anastomosing and arborising vascular channels lined by single layer of flattened spindle-shaped cells with scant cytoplasm; lumina have a paucity of erythrocytes
Leiomyosarcoma		Smooth muscle	Long, thin mesenchymal cells arranged in aggregates or linear bundles; nuclei elongated or cigar-shaped.

2.4 Causes of soft tissue sarcoma development

There are sporadic cases reported in which dogs have developed a STS in association with known previous injuries, parasitic infections (e.g. *Spirocera lupi*), implants and trauma.[15-18] However, for the vast majority of canine STS, the cause of tumour development is unknown.

In humans, analysis of the molecular genetics of STS has divided sarcomas into two main categories: (i) sarcomas with defined genetic alterations including specific chromosomal translocations and oncogenic mutations; and (ii) sarcomas with a "complex genomic profile" which may involve dozens of molecular abnormalities.[19, 20] The majority of STS seem to have the latter characteristic which suggests there is no common mutational pathway for development. This suggests tumour initiation and progression may be a random event, possibly exacerbated by the increased mutagenesis that can accompany localised areas of chronic inflammation.[21, 22]

2.5 Clinical features of a soft tissue sarcoma

There is no apparent breed disposition for STS, but middle-to-large breed dogs tend to be more commonly affected.[2-5] There is a variable ratio of males and females affected in different reported populations but overall, the sex or neuter status of the dog appears to have little, if any, influence on disease development or progression. Affected dogs tend to be middle-aged or older, with the median age at diagnosis reported to be between 10-11 years (range, 2 - 16 years).[3, 5, 13, 23, 24]

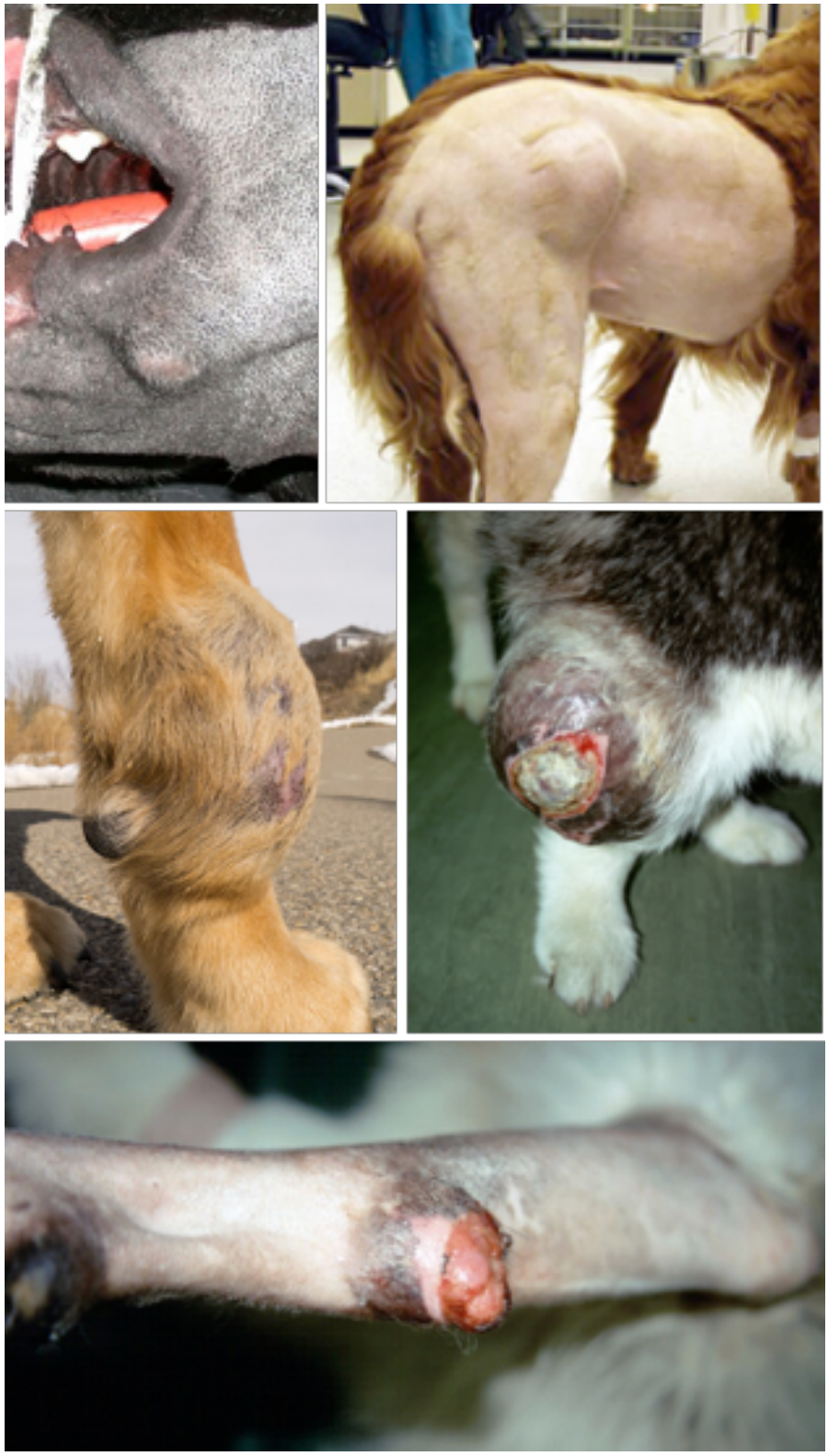
Soft tissue sarcoma are usually firm, plump masses that have expanded under the overlying skin (Figure 2.1). Tumours are most commonly located on the limbs, a location that may represent up to 60% of cases.[3, 5, 10] The trunk (including the tail) is involved in about 35% of cases and the head in 5% of cases. They are not usually painful or associated with any notable discomfort for the patient. On palpation, the STS may appear to be firmly adherent to the underlying tissues, but in other cases the tumour can feel quite mobile and on palpation may wobble within the subcutaneous tissue.

Clinical signs associated with the mass may be influenced by location. In most cases, there are no clinical signs evident apart from the palpable mass. However, if a large mass is deeply located within the muscles or is closely associated with a joint, it may interfere with musculoskeletal function. In these cases, a mechanical lameness may be the only presenting sign with the mass itself occult to palpation.

2.6 Diagnosis

The initial diagnostic investigation of a cutaneous or subcutaneous STS is usually by cytological evaluation of samples obtained by fine needle aspiration.[3, 5] Care is required with sample collection and interpretation because mesenchymal cells do not exfoliate readily during fine needle aspiration. In addition, the neoplastic cells of a STS can appear similar on cytology to reactive mesenchymal cells that could be present within an inflammatory lesion;[25] this similarity can lead to both false-positive and

Figure 2.1:
Soft tissue sarcoma are a heterogenous group of tumours with a variety of clinical presentations. The physical characteristics of the tumour including size, mobility and location can impact on treatment strategies and prognosis.



false-negative results on cytology so interpretation needs to be performed in conjunction with the physical characteristics of the mass.[26] When compared to the final histologic diagnosis, cytology correctly identified a mass to be a mesenchymal tumour in 97% (67 of 69) cases, but a diagnosis of STS was accurate in only 67% (45 of 69) cases.[26]

To achieve a more confident diagnosis that a palpable mass is a STS, incisional biopsies can be taken with a scalpel, biopsy punches, needle core biopsy instruments, or trephines.[27-29] Biopsies can be obtained under local anaesthetic, sedation or general anaesthesia depending on the temperament of the patient or the location of the tumour.[29] When only small sections of the tumour are obtained, multiple samples should be obtained from different locations to improve the diagnostic yield. The biopsy tract should be located where it can be removed at the time of definitive surgery, and care should be taken not to breach the lateral or deep boundaries of the tumour as this could allow the tumour to invade beyond its original location.[28] As well as giving more descriptive microscopic information about the mass, an advantage of an incisional biopsy is it can be used to determine the grade of the tumour. The tumour grade can, in turn, be used to infer some clues about the likely aggressiveness of the tumour.

2.7 Tumour grades in soft tissue sarcoma

A tumour grade – ranging from low (grade 1), intermediate (grade 2), or high (grade 3) – can be assigned to a STS based on various histological criteria, as outlined in Table 2.3. The histological grade of a STS is currently one of the

most validated criteria to predict outcome following surgery in canine patients.[9, 11] Higher tumour grades are associated with a more aggressive biologic behavior, which translates to increased rates of local recurrence, distant metastasis and shorter disease-free intervals.[9, 11, 13]

Low grade (grade 1) tumours are the predominant form in veterinary medicine, but published incidences may be influenced by the source of affected patients. In studies reporting on tumours sourced from first opinion practices, low-grade tumours predominate (51-84%) and high-grade tumours tend to be uncommon (7%).[9] However, in studies derived from referral practices, high-grade tumours are more commonly represented with proportions from 22.7 to 29% reported.[11, 12]

Given the importance of tumour grade in determining subsequent behaviour of the tumour, it would seem sensible to try and determine the grade of the STS prior to performing surgery. Unfortunately, the accuracy of pre-treatment biopsies at determining grade is currently limited. In one study, 29% of pre-treatment biopsies under-estimated and 12% over-estimated the final histological grade assigned to the tumour after resection.[30] This study concluded that while pre-treatment biopsies are relatively accurate at distinguishing high from low-grade sarcoma, a pre-treatment result of 'low-grade' should be viewed with caution. This lack of accuracy of pre-operative analysis of the tumour is frustrating as one of the prime reasons for performing pre-operative interrogation of the STS would be to identify the low-grade subset that are known to be less aggressive in their behaviour.

However, if the biopsy actually under-estimates the grade of the tumour in almost a third of cases, performing a more conservative treatment may be inappropriate if the tumour is subsequently determined to be higher grade as this would increase the risk of treatment failure. This inconsistency needs to be resolved to allow pre-operative interrogation of a tumour to be more accurate and more commonplace.

2.8 Staging of soft tissue sarcoma

A staging system has been described for STS in dogs, and takes into account the size of the tumour, the involvement of local lymph nodes, and the presence of metastasis.[31] This staging system was modified from the system reported for human STS, developed by the American Joint Committee on Cancer. Modifications to the canine STS staging system have been published,[5] with inclusion of whether the tumour is located superficially or deep within the tissues due to similar modifications to the staging system for human STS. However, neither the original nor the updated versions of the canine staging system have ever been correlated with patient prognosis in clinical trials.

Soft tissue sarcoma are thought to metastasise by a haematogenous route so secondary tumours are most likely to develop in the lungs; metastasis to the regional lymph nodes is rare.[11] Based on general oncologic principles, three- or four-view thoracic radiographs (or computed tomography (CT)) should be considered prior to surgery to check for pulmonary metastasis.[3]

Table 2.3:
Grading System for Cutaneous and Subcutaneous Soft Tissue Sarcoma in the Dog (from Dennis et al.)[13]

Differentiation		Score
	Sarcomas most closely resembling normal adult mesenchymal tissue, by type (e.g. well-differentiated perivascular wall or peripheral nerve sheath tumours, well-differentiated fibrosarcomas or well-differentiated liposarcomas)	1
	Sarcomas for which histologic type can be determined, although differentiation is poor (e.g. poorly differentiated liposarcoma, fibrosarcoma, poorly differentiated perivascular wall tumour or peripheral nerve sheath tumour)	2
	Undifferentiated sarcomas, sarcomas of unknown type	3
Mitotic index		**Score**
	0–9 mitoses per 10 high-power fields (400x)	1
	10–19 mitoses per 10 high-power fields (400x)	2
	>19 mitoses per 10 high-power fields (400x)	3
Tumour necrosis		**Score**
	No necrosis	0
	<50% necrosis	1
	≥50% necrosis	2
Histologic grade: (total score is a combination of scores for differentiation, mitotic, and tumour necrosis) Grade I = ≤3 Grade II = 4–5 Grade III = ≥6		

Abdominal ultrasound may also be considered to allow detection of metastasis to the liver, kidney or other parenchymous organs. In reality, the incidence of detectable metastasis at the time of surgery for STS is low. In

one study that examined either 3-view inflated thoracic radiographs collected under general anaesthesia or thoracic CT scan, metastatic lesions were found in about 10% (16 of 131) of dogs with STS at the time of presentation for surgery; this included 5 dogs with grade 1 STS, 2 dogs with grade 2 STS and 9 dogs with grade 3 STS.[32] The presence of metastasis increased with the known duration of the mass; for STS that had been present for >3 months, the risk of metastatic disease increased by a factor of three.[32]

While STS are generally considered to have a low to moderate rate of metastasis developing in the months and years following removal of the primary mass, this is not well documented. Different authors have described metastatic rates ranging from 1.7 – 41%,[1, 11, 12, 33, 34] but this data is derived from retrospective studies so will be influenced by the scrutiny of investigation. Further analysis of the importance of staging in the prognosis of canine STS is detailed in a later section of this chapter.

2.9 Treatment of soft tissue sarcoma

Surgery is considered the most important therapy for the management of canine STS. Radiotherapy (RT) and chemotherapy can also play a role in the control of secondary metastasis and local recurrence; however, these modalities tend to be relatively ineffective in the treatment of a grossly visible tumour. Current advice using each of these modalities is outlined in further detail in the following sections.

2.9.1 Surgery

Surgical strategies for STS are defined by the extent of resection about the gross boundaries of the tumour. These are based on the surgical margins that were described for the management of musculoskeletal tumours in humans.[35]

The types of resection that have described for canine STS include:[5, 13, 23]

- **Intra-tumoural**: when the capsule surrounding the tumour is penetrated and the gross tumour removed, often in a piecemeal fashion
- **Marginal**: when the tumour is excised at the level of the pseudocapsule, or when the mass is 'shelled-out' from the surrounding tissues
- **Wide**: when the tumour is excised with a cushion of normal tissue about all boundaries. The width of this tissue cushion may be anywhere from 1-50mm, or more. Some papers have also described a subclassification of **Narrow** when the width of tissue removed about the mass is small; in this instance the width of the tissue cushion is ill-defined, but may be about 3-5mm
- **Radical**: when an entire anatomic segment is removed e.g. amputation

For wide and narrow resections, an *en bloc* surgical technique is performed. The *resection margin* is defined as the amount of skin and tissue removed by

the surgeon around the entire circumference of the tumour. An *en bloc* resection is performed by measuring the desired resection margin about the gross perimeter of the tumour with a ruler. Incision of the skin and subsequent dissection of the underlying tissues is then performed, with care taken to maintain this defined resection margin about the entire circumference of the tumour. The resection extends perpendicularly through the skin, subcutaneous tissues, and muscles until an appropriate deep margin is identified and penetrated. Muscles, non-vital nerves and blood vessels that branch into the resection field are cut about this measured lateral border of the surgical margin effectively isolating a 'block' of tissue ready to be elevated from the wound.

Incision through a tissue boundary deep to the STS allows the tumour to be removed from the body in one contained piece. Identifying a defined deep boundary is an important aspect of the surgery. It is not normally possible to take a measured quantity of tissue from the deep boundary that is similar to that obtained from the lateral boundaries without encountering vital components of the skeleton or other internal organs. For this reason, the surgeon will make use of tissue barriers that are considered resistant to tumour invasion. Such tissue barriers include muscle, fascia, joint capsule, cartilage, or bone.[35] The importance of these tissue barriers is based on the recognition that during growth, STS tend to preferentially expand within the tissues along a path of least resistance. Cellular invasion will be constrained by these tougher tissue boundaries.[36] Some STS are capable of invading beyond these barriers, but the invasive path is usually via perforating vascular channels or surgically created openings, such as a biopsy tract.

Invasion through a tissue barrier may not occur until late in the disease course when the internal pressures of the expanding mass start to exceed the resistive pressures of the tissue barrier.[36]

When considering resection margins in STS, it is important to recognise the importance of not only the *width* of the surgical margin relative to the pseudocapsule and surrounding reactive zone, but also the *quality* of any defined anatomical barriers around the tumour. The width of the surgical margin obtained about a STS has long been considered a factor important for effective control of the tumour. One of the first studies to publish the outcome of dogs treated for STS revealed overall rates of local recurrence of 44% and median survival times (MST) of less than 2 years.[1] In the same study, STS with a mitotic index (MI) of 9 or more did poorly with local recurrence rates exceeding 60% and MST of less than 1 year. These authors concluded that radical surgery, including limb amputation, was justified given these poor rates of local control. A subsequent study used *en bloc* surgical margins incorporating a minimum of 3 cm of normal tissue laterally and one clean fascial plane deep to the tumour;[11] these margins were similar to those described for the surgical management of human STS at the time.[37] This study achieved recurrence rates of less than 15% and MST of 1416 days – almost twice that of the original study. This paper was from a highly respected institution that had a pioneering influence on the emerging discipline of veterinary oncology so the resection margins utilised in this paper soon became the accepted standard for the management of all STS.[2, 3, 5, 11]

Despite the favourable results reported after wide excision of STS, obtaining these excision margins may not always be an option in every case. Lateral margins of 3cm about the gross boundaries of the mass may be achievable when the tumour has arisen on parts of the body (e.g. the trunk) where adequate skin redundancy is available for wound closure or reconstruction. However, more than 50% of STS arise on the appendicular skeleton.[11, 24, 38] Wide resections of tumours on the limb increase the risk that vital nerves, vascular structures, significant muscles or tendinous structures become involved in the resection, which may result in unnecessary morbidity or dysfunction. Other patient comorbidities or the financial constraints of the client may also prevent extensive surgery being performed. Several authors have challenged the requirement for wide surgical excision margins,[10, 24, 39] with some studies suggesting the extent of resection performed did not influence the disease-free interval or overall survival.[9, 23, 24] This debate remains unresolved. In the second part of this Chapter, some of the issues and controversies that surround the role of surgical margins in the management of canine STS will be discussed in more detail.

Different qualities of tissue are also believed to resist STS invasion to a variable extent, causing some surgeons to characterise different tissue layers as providing either a 'thick' or a 'thin' barrier.[40] A thick barrier is defined as a physically strong membranous tissue such as bone, joint capsule or structural fascia (e.g. the fascia lata or lumbar fascia), while a thin barrier is typified by weaker membranous tissue such as muscle, connective fascia, periosteum in adults, epineurium, etc. In an effort to standardise the surgical management of human STS, 'normal tissue equivalents' were assigned to

different tissue barriers: thus, a thick barrier was considered equivalent to 3cm of normal tissue and a thin barrier was converted to 2cm of normal tissue.[40] Joint cartilage was considered to be the equivalent of 5cm of normal tissue. During removal of a STS, a surgeon working to these guidelines would attempt to remove the mass with a total of 5cm of tissue equivalents which would represent a combination of normal tissues, as well as thin and thick barriers. Because tissue barriers can be considered to help constrain invasion by the STS, there can be occasions where removal of an entire anatomical compartment is preferable to a measured *en bloc* resection margin. For example, a STS will occasionally arise within an individual muscle or muscle group. In those cases, removal of the whole "compartment" of tissue - which may include a single muscle or group of muscles that surround the STS, removed from origin to insertion - may provide a superior outcome even if the actual measured quantity of tissue bounding the tissue is less than the 3 to 5 cm generally recommended.[36, 41]

2.9.2 Radiotherapy

Radiotherapy uses high-energy x-rays to disrupt cellular DNA, which ultimately causes apoptosis of the affected cell. Because cancer cells tend to have poor DNA repair mechanisms, RT has become an important modality for the treatment of many different types of cancer. For the management of cancer, RT may either be used in place of surgery or may be combined with surgery to allow resection margins to be reduced without compromising overall treatment success. In human STS management, RT is now considered an integral component of effective local therapy for STS, and has enabled a

reduction in resection margins without compromising local control rates.[42] Radiotherapy may be employed either before (neoadjuvant) or after (adjuvant) surgery. While there is no proven difference in disease outcome according to treatment sequence,[43-45] the negative impact of neoadjuvant radiation on wound healing can influence the decision on a case-by-case basis.[45]

In veterinary patients, outcomes from curative-intent adjuvant RT after incomplete STS resection have been reported. Using hyperfractionated protocols of either 42 to 57 Gray (Gy) at between 3 - 4.2 Gy daily [46], or 63 Gy delivered in 3 Gy fractions on alternate days,[47] overall MSTs of 1,082 - 2,270 days and local recurrence rates of 16 - 31% have been described. The study with a higher rate of local recurrence included 8 of 37 (22%) dogs with oral mesenchymal tumours which will negatively bias these results as oral fibrosarcoma are not considered to be STS and are known to have high rates of local recurrence.[46] A more recent study reported on the use of hypofractionated RT after planned marginal resection of STS in dogs.[34] The treatment protocol used in this study consisted of four weekly doses of RT (6-9 Gy per dose) to a total treatment dose of 24-36Gy.[34] In that study, local recurrences developed in 18% of dogs, with metastatic disease occurring in 9%. Follow-up periods were 426-2035 days (median 1339 days). Although these studies suggest that using RT to treat surgical wounds where microscopic STS is known to persist can provide adequate local control in most cases, the actual efficacy of RT is difficult to assess from these papers. No control group was included in any of the studies, with outcomes compared to historical controls only. The premise for giving RT treatment

when microscopic tumour cells are detected at the resection margins is to try and prevent the inevitable regrowth of the tumour; however, as will be discussed in later section of this chapter, this inevitability is by no means assured and local recurrence may develop in less than 30% of dogs with incomplete margins.[9, 11] The potential for recurrence will also be affected by the grade of the tumour, with low grade tumours less likely to recur even when resection margins are incomplete.[9] Existing studies using adjuvant RT report recurrence rates of between 16 and 31%. In two of these studies, cases were derived after surgery was performed in first opinion practice, so there would have been a bias for more low-grade tumours to be represented.[46, 47] It is entirely possible that current RT protocols reduce the risk of recurrence by only a few percentage points. Further veterinary studies are required to enable better understanding of which patients are most likely to benefit from RT, with prospective and randomised trials performed to allow the true benefit of different RT protocols to be determined.

2.9.3 Chemotherapy

The value of chemotherapy in veterinary patients remains unclear and robust evidence is limited.[5] Only one study has been published evaluating the effect of adjuvant doxorubicin (30mg/m^2) in high-grade STS; rates of local recurrence, metastasis and overall survival were similar to those observed in a historical population.[48] However, this paper described the outcomes for only 39 dogs that were managed almost 20 years ago. No control population was included, with outcomes compared to a historical population that had

been treated with surgery only more than 10 years previously. Both populations were analysed retrospectively, and the thoroughness of evaluation of each patient during the study period is uncertain. Other publications have described outcomes for dogs with STS using other chemotherapy drugs such as mitoxantrone or ifosfamide;[49, 50] these papers reported outcomes for a broad range of tumour types with a mixture of inclusion criteria and no control populations, so it is difficult to draw conclusions on their true efficacy in the prevention of local recurrence or metastasis in dogs with STS.

In humans with STS, where development of metastatic disease remains the most significant cause of tumour-related death, the role of adjuvant chemotherapy (typically doxorubicin based) is also controversial. Results from two large meta-analyses have shown either no response,[51] or at best a small but significant benefit for local recurrence (Odds ratio (OR) 0.73; 95% CI, 0.56-0.94; P = 0.02), distant recurrence (OR 0.67; 95% CI, 0.56-0.82; P = 0.0001), and overall survival (OR 0.67; 95% CI, 0.56-0.82; P= 0.0001).[52] Currently, adjuvant systemic chemotherapy is not given routinely for every human patient with a STS but may be incorporated into the treatment plans for high-grade tumours or for specific histologic types.[53]

Because local recurrence of a STS, as opposed to metastatic disease, is considered the most common cause of tumour-related death in dogs, the use of different forms of chemotherapy to try and slow or prevent local recurrence after surgery has been investigated. In this context, low-dose continuous chemotherapy – or metronomic chemotherapy (MC) – has

received interest.[54, 55] Rather than being directly cytotoxic, MC is thought to inhibit tumour growth via a combination of anti-angiogenic and immunomodulatory effects.[56] One study compared 30 dogs treated with MC with 55 historical controls. This paper suggested that the use of MC led to significantly improved disease free intervals ($p<0.0001$).[54] However, selection bias in the control population may have skewed the conclusions of this study. In this study, the treated population included 30 dogs that were started on MC when histologic assessment had confirmed an incomplete resection margin. Although not explicitly stated, the outcomes of this treatment group was compared to a control population of 55 dogs where gross recurrence of the STS had occurred following surgery; these 55 dogs were derived from a total population of 1311 dogs treated for STS, representing a 6% overall recurrence rate. The recurrence rate in the treated population is not stated; from a visual interpretation of the Kaplan Meier curve it is estimated to be 4 of 30 dogs (13%). Because the end-point of this study was disease-free interval rather than the incidence of recurrence, the impact of this apparent selection bias is hard to predict; the presented data would suggest that treatment with metronomic chemotherapy significantly slows the development of local recurrence.[54] However, given the large discrepancy in population size between the treated and control groups where recurrence occurred, it is likely the observed difference in disease-free interval may be spurious. Further investigation is required to determine whether metronomic chemotherapy is effective in preventing or slowing the development of local recurrence in STS.

2.10 Prognosis

The prognosis for the majority of dogs with STS is generally good if a complete resection can be achieved.[9, 11, 13] However, local recurrence can develop in up to 75% of dogs.[1, 10-12, 23, 24, 38, 39, 48] Rates of metastasis are less well-defined but may develop in between 1.7% and 41% of cases.[10, 11, 13] Overall, about 20 to 30% of dogs will ultimately die of their disease.[9, 11, 12, 24, 39, 48, 57, 58] Continued efforts to improve management options and to recognise those dogs at risk of recurrence and death remains important.

In reality, the owner of a dog with a STS doesn't want to hear that the prognosis for their dog is "generally good", or that there is a 20-30% chance that their dog will die from the tumour despite treatment. They want to understand what the prognosis is for their own individual dog, and whether treatment will be successful in ensuring the tumour does not come back. The answers to these more specific questions are harder for a clinician to answer. Cancer is a heterogenous disease, and one individual tumour will not present with the same characteristics as another. Variations in tumour size, location and other patient factors will impact on the ability of a surgeon to remove the mass with an appropriate cushion of healthy tissue. The generalised prognostic figures quoted above are also derived from retrospective analyses of clinical cases performed at different institutions, with different surgeons working under different conditions in different geographic locations around the world. These differences introduce bias and limitations into the case selection and application of surgical strategies that may impact on the

characteristics of the tumour population being operated on, and the consistency of clinical management between different studies. Being retrospective in nature, determination of patient outcomes and the rates of local recurrence, metastasis and survival have the potential to be imprecise as relevant data may not have been collected in the first instance, or is reliant on recall or secondary knowledge. Finally, almost all of the studies have involved sample sizes of less than 100 animals, which restricts the statistical power necessary to correctly identify clinical features that may be influential in outcome.

In light of these limitations in the existing literature, it is understandable that controversy exists into whether certain characteristics of a STS may influence the outcome of patients more than others, or whether particular treatment strategies are more effective than others. In the next section of this chapter, the evidence for some of the prognostic factors that may influence the outcome of STS in the dog will be examined. Comparative evidence from human STS will also be explored: this is because a similar debate on the factors that influence the management of STS has occurred in the human literature,[36, 59-61] and many of the treatment challenges posed by this tumour in humans are comparable to those that confront the veterinary surgeon. The individual prognostic factors that will be examined in the following section include: the histological type; histological grade and other known markers of proliferation; tumour size, location and palpable characteristics; the presence of metastasis; and finally the importance of surgical margins, including how resection margins are evaluated and the evidence to support what an appropriate width of resection margin is

required. To conclude, the importance of STS structure and how the tumour microenvironment may influence whether an individual STS may recur after surgery will be reviewed.

2.10.1 Histologic type

As discussed previously, all subtypes of STS have traditionally been considered as a single group for prognostic purposes largely because differentiating individual tumour types by light microscopy can be unreliable.[13] However, in human STS, there is increasing evidence that individual subtypes may exhibit differences in local invasiveness, metastatic potential and recurrence.[62, 63] In current studies on canine STS, any evidence for differences in outcome between various histologic subtypes is limited by small population sizes or a lack of rigour in histological diagnosis.[13] There is a need to develop better tests that allow individual subtypes with variances in clinical behaviour to be identified with more confidence. This may require the increased use of immunohistochemical markers, or even molecular profiling.[13]

2.10.2 Histologic grade

Histological grade is considered the most important prognostic factor in human STS,[64-66] and is also one of the most validated criteria to predict outcome following surgery in canine patients.[9, 11] In one study, the histologic grade of a STS was found to be a strong predictor of local recurrence after surgery with recurrence rates for low, intermediate and high-grade tumours varying from 7, 34 and 75 percent respectively.[9] The

findings of this paper are important, as it demonstrates a correlation between tumour grade and different rates of local recurrence for a cohort of STS that had all been resected with narrow margins. As discussed previously, current surgical advice for STS was derived from evidence generated from cases treated in referral or academic practice.[11] However, cases managed in referral practice are a selected population; they have been referred for treatment at a specialist centre either because their STS was showing a more aggressive clinical appearance (e.g. large size, recent rapid growth, or a fixed and immobile characteristic) or were located in locations that made surgery more challenging. Because of this selection bias, interpreting the prognosis for patients in response to certain treatments needs to take into the account the population pool from which the treatment cohort was derived. Outcomes are likely to be better in those studies with a higher proportion of low-grade tumours,[9, 23, 24] compared to studies with more high-grade tumours.[1, 10-12, 38] It follows that treatment advice may need to be stratified according to the grade of the tumour.

It is also well-recognised that the grading of tumours is subjective and variation in interpretation between different pathologists has been reported for STS and other tumour types.[67, 68] In one study on canine STS, the assigned grade or diagnosis of a mesenchymal tumour was modified in 5 out of 15 cases (33%) following review of the slides by a second pathologist.[68] In two of these cases, this revision led to an increase in grade (from grade 2 to grade 3), while in another two cases, the interpretation changed from a malignant mesenchymal tumour to a benign disease. In the final case, the diagnosis was modified from an oral sarcoma to a melanoma. These changes

have the potential to alter the potential prognosis for these patients. When the original histologic assessment under-estimated the aggression or metastatic potential of the tumour, these dogs may have been denied consideration for adjuvant therapy that could have prevented or slowed tumour recurrence. For the dogs diagnosed with a malignant neoplasm when their tumour was actually benign, their outcome would obviously be better than expected. However, these dogs may have been subjected to treatments in excess of that needed for their underlying disease. The impact of this high error rate for an important prognostic indicator like tumour grade has implications not only on the management for an individual dog, but also on the ability to interpret the treatment recommendations from existing literature. Development of more objective predictive markers that correlate reliably with tumour behaviour would be important to help support clinical decision making.

2.10.3 Mitotic index and other proliferative markers

As a measure of proliferative activity within the tumour, the MI can provide additional prognostic information about an individual tumour.[13] An MI of more than 9 mitotic figures per 10 high power fields (hpf) has been associated with increased (and earlier) rates of tumour recurrence, higher rates of metastasis and reduced overall survival in several studies.[1, 9, 11, 13, 69] With an MI ≥9, MST range from 150 – 343 days, compared to 826 – 1138 days with an MI <9.[1, 70]

The histologic determination of MI is actually a single 'snap-shot' of the proliferative activity of cells frozen in time at the time of tumour fixation. The

use of various proliferative markers, such as Argyrophilic Nucleolar Organizer Region (AgNOR) and the Ki-67 protein, can provide additional information about the mitotic activity of a tumour as they detect chemical signals that may persist within the cell across the whole mitotic cycle.[69] In canine STS, increased AgNOR and Ki-67 scores have both been associated with reduced survival time and correlated with tumour grade and MI.[69] However, the use of these markers has not been routinely adopted in the evaluation of canine STS.

2.10.4 Tumour size and growth rate

Several canine and human studies have suggested that tumours larger than 5cm (golf-ball sized) have shorter disease-free intervals or survival times.[11, 64, 65, 71-74] However, other authors have not found any association between tumour size and outcome.[1, 24, 57] A STS with a history of sudden or rapid growth, or the presence of tumour necrosis and ulceration, has also been suggested to imply a more aggressive growth characteristic,[5] but this has not been validated in clinical trials. There may be confounders between tumour size and other prognostic factors that may influence outcome. Larger tumours may be more difficult to remove and may be more likely to impinge upon vital anatomical structures, which thus limits the ability to maintain an appropriate resection margin about the entire tumour. Soft tissue sarcoma resected in first opinion practice also tend to be smaller than those managed in referral practice,[11, 24] so the source of the tumour population also needs to be considered.

2.10.5 Palpable characteristics of the tumour

Most STS are readily palpable and may appear to be quite discrete and encapsulated. Other tumours may be multi-lobulated, soft and have very indistinct borders. Although the superficial aspects of the mass may appear quite mobile, the base can be indistinct and potentially attached to underlying bone or fascia.[5] This difference in mobility between different tumours may be significant in terms of prognosis; tumours that feel more 'fixed' to underlying tissues have significantly decreased disease free intervals ($P<0.0001$) and survival times ($P=0.007$).[24] It is hypothesised that more adherent tumours may have a different tumour microenvironment that causes them to be more infiltrative or enables greater migration of tumour cells into the periphery.[75] However, interpretation of tumour mobility is a highly subjective feature and the prognostic significance of this finding has been inconsistently reported by other authors.[13] This clinical finding needs to be validated in a prospective setting to see if it can help consistently predict prognosis.

2.10.6 Presence of metastases

The metastatic rate for dogs with soft tissue sarcoma has been reported to be between 1.7 and 41%.[10, 11, 13] The published metastatic rate for grade 1 and 2 tumours is usually low, with most studies reporting incidences of less than 15%.[11, 38, 47, 76] For high grade tumours, the quoted figure is consistently higher and may be as much as 44%.[11, 48, 69] Other authors have reported intermediate levels of metastasis for grade 2 tumours, with rates between 27% and 33%.[69, 70] Metastasis is five times more likely when tumours

have a mitotic rate of 20 or more.[11] Other factors that have been associated with an increased risk of metastatic disease include the percentage of tumour necrosis and local tumour recurrence,[5] although this latter characteristic is inconsistently reported. The accuracy of all of the data relating to STS metastasis is uncertain. Determination of metastasis is largely reported from retrospective studies, so there will be considerable bias and variation in the intensity of investigation for the presence of metastatic disease. Metastasis may not develop until many weeks or months after surgery so the period of follow-up of patients since surgery will affect the reported incidence; in one study, the median interval from surgery to detection of a metastatic lesion was 365 days.[33] In many studies, no histological confirmation of metastatic disease was performed, and a diagnosis of metastasis was reliant on imaging findings only.[13] This raises the possibility that a newly discovered metastatic lesion may not necessarily be due to the previously resected STS; the majority of dogs with STS are elderly, so it is possible that some of these dogs could develop a new primary malignancy that may be occult to examination.

2.10.7 Resection margins

Wide resection of STS has long been considered an important requirement if adequate local control is to be achieved. In the first veterinary paper to describe outcomes for dogs STS, local recurrence developed in 25 of 103 (34%) of patients with MSTs of less than 2 years.[1] This paper does not specifically state what resection margins were used about the tumour, other than stating the "the mass was resected with as much surrounding normal

tissue as permitted by the site". This paper was published at a time when veterinary oncologic surgery in the UK was in its infancy, so it would seem unlikely that extensive resection margins of more than 1cm will have been attempted at that time. The next paper on STS was not published until 17 years later and came from a respected oncologic centre in the USA. By that time, the importance of oncologic principles were becoming realised, using comparative evidence from human oncology.[77] In this paper, the STS were managed with wide resection margins that included 3cm lateral to the tumour and a deep fascial plane; these margins were based on the resection margins being described for human musculoskeletal tumours.[35, 36] Local recurrence was observed in 11 of 75 (15%) dogs,[11] with a median survival was almost 4 years. Subsequent studies where wide resection margins were used appeared to validate this finding, with local recurrence rates of 0 of 19 (0%),[76] 4 of 54 (7.5%),[38] and 10 of 50 (20%)[78] dogs. Some studies showed that wide surgical margins were more likely to achieve complete tumour removal than marginal or narrow resection,[10, 38] or that recurrence was more common with a narrow or marginal excision.[24] However, statistically significant correlations between resection margins and local recurrence have not been determined.[24, 38] Radical excision has not been shown to improve survival times when compared to patients with other resection margins.[11]

More recently, there have been several studies that have challenged the necessity of wide resection margins to minimise the chances of STS recurrence. Local recurrence rates of just 10.8% (follow-up 210-2202 days) were reported in 35 dogs with low-grade spindle cell tumours of the

extremities treated by marginal excision only.[23] In another prospective clinical study, 100% local disease control and 93% one-year disease free interval was achieved in 14 dogs with 1cm lateral resection margins and a single fascial plane beneath the tumour.[10] However, in that paper, patient follow-up times were only 12 months, which is inadequate for soft tissue sarcoma. In another paper examining outcomes for dogs with STS treated exclusively in first opinion practice, local recurrence rates of 20.8% were reported, despite marginal or narrow resections being performed in 77% of cases.[24] The median follow-up in that paper was 785 days.

Overall, it must be concluded that a relationship between resection margins and overall survival or local tumour recurrence has not been demonstrated in the existing literature. Moreover, the quality of any such evidence, even when it is present, must be considered poor due to the effects of numerous confounders, including tumour size, location and grade. Although the size of the resection margin is a metric that may be of greatest immediate relevance to the surgeon, it is probable there are too many variables that influence the relevance of such a macroscopic measurement, and other prognostic markers may be of more relevance.

2.10.8 Margin evaluation

Irrespective of the actual width of resection margin performed, demonstration of a histological margin that is clear of tumour cells – described as a "histological tumour free margin (HTFM)" is considered the best predictor for improved local tumour control of a STS.[13, 79] When neoplastic cells are seen immediately adjacent to the resection margins when

examined using histology, tumour recurrence is more than ten times (95% CI 1.33-82.42) more likely to occur.[11]

Because of the association with increased local recurrence, obtaining a resection margin that is free of tumour cells on histological assessment is considered by many surgeons to be the ultimate goal of oncologic surgery.[80]. However, the histological assessment of surgical margins as an indicator of complete tumour removal *in all planes* can be highly flawed, either as a consequence of processing methodology or the practical realities of a commercial laboratory service.[13, 25] An important limitation of margin evaluation is that only a small fraction of the overall tumour circumference can be examined microscopically; most commercial veterinary laboratories evaluate neoplasm specimens using between three and six tissue sections. Pathologists therefore need to be strategic in assessing which sections of a large tumour bulk to evaluate.[25] Recommendations have been published to improve consistency in histologic processing and reporting.[25]

Aside from the practical limitations that prevent evaluation of the entire tumour circumference, there are other technical factors that can influence the accuracy of margin evaluation. Due to the effects of tissue elasticity and the deformation that occurs from the effects of fixation in formaldehyde and subsequent processing steps required to get a section of the tumour onto a microscope slides,[13, 25] the final measured histologic margin of tissue surrounding the visible tumour boundary can be 35% to 42% smaller than the original measured surgical margin.[81] The extent of distortion has been found to differ for different tissues (e.g. skin, muscle, fat), based on their lipid

content.[82] Contraction of tissues will also differ between tumours, probably due to variances in stromal characteristics and microscopic infiltration about the tumour boundary.[81, 83] There is a compounding effect on specimens composed of a tumour and multiple tissue types (e.g. skin, subcutaneous, or musculoskeletal tumours) that causes different tissue layers to distort and twist.[25] Further distortion of the tissue will occur during histologic processing; this is due to the effects of alcohol and xylene washing that prepares the tissue to be infiltrated and embedded in paraffin wax, and the fragmentation that can occur during microtomy and mounting on a slide.[84] Due to the combined effects of these influences on tissue dimensions from excision to final interpretation on a microscope slide, the final measured histologic margin of tissue surrounding the visible tumour boundary was found to vary between 43% and 176% of the original measured surgical margin.[81, 82] This work suggests that the measured HTFM may actually bear little relevance to the actual surgical margin obtained; the histologic measurement of a tumour margin can under- or overestimate the actual extent of the tissue barrier that was maintained about the tumour during excision. Due to this variability in tissue shrinkage and deformation between patients, tissue and tumour types, extrapolating an optimal surgical resection margin from a desired HTFM will not be possible.

Another limitation to the accuracy of margin assessment is the ability of the histologist to reliably identify fascial tissues as a distinctive structure. As discussed previously, a defined fascial boundary is widely acknowledged as a crucial aspect of the deep resection margin.[2, 11] While a fascial layer may appear distinct to the surgeon, the same structure is often difficult to identify

microscopically. From an oncologic perspective, if the pathologist cannot confidently recognise the fascial tissue that the surgeon utilised as a distinctive barrier during resection, the histological appearance of the deep margin may be interpreted as just a few cell layers of tissue, which raises concerns for an incomplete resection. Ultimately, the surgeon needs to interpret the histological findings in conjunction with the knowledge of their surgical plan. The surgeon knows best whether a thick or thin fascial barrier was utilised at any part of the dissection, which sections of the tissue appeared concerning at the time of surgery, or where margins were compromised due to proximity with vital anatomical structures. Coloured inks can be used to paint lateral and deep margins of the excised tissue.[25] Inking can help overcome the difficulties in margin evaluation that occurs when different tissue layers become distorted during fixing, as the pathologist is able to observe the inked margin on the microscopic specimen. If tumour cells are seen to abut the section of tissue inked by the surgeon, there can be more confidence that this resection margin may be incomplete.[25]

The precise width of HTFM necessary to completely eliminate recurrence has not been investigated in the dog. Different studies use different criterion to define a HTFM width that equates to either a “complete margin” or “incomplete margin”, or often fail to describe one at all. When it is described, the widths of normal appearing tissue about the pseudocapsule may range from 1mm to 10mm.[9, 10, 23, 48, 76] These inconsistencies in the definition of what extent of HTFM is required to ensure complete excision of the STS makes it challenging to compare the outcomes from different clinical studies

based on different sizes of gross resection margin.[13] In one study, no recurrences were observed when a HTFM of >3mm was found between the tumour and surgical margins on histological review,[85] but this paper was limited to dogs with low grade spindle cell tumours only. Another study showed no local recurrences in 30 dogs with a HTFM of ≥1mm;[9] while no influence of tumour grade was detected, this study was performed on cases submitted from first opinion practice so high-grade tumours were uncommon, representing only 7% of the study population. Only one paper has so far demonstrated a statistically significant correlation between HTFM (>2mm, in this case), local recurrence ($p<0.001$) and disease free interval ($p = 0.004$);[86] this was a study of 20 dogs with STS with 30% grade 1 and 70% grade 2 and 3 tumours. In all other studies where a HTFM was reported, interpretation of significance was affected by either inadequate case numbers,[10] inadequate follow-up times,[11, 76] or the inclusion of dogs undergoing re-excision of a recurrent STS or surgical scar.[12, 57] In two studies, no correlation between HTFM and local recurrence was evident.[9, 38] Once again, the current literature provides inadequate or insufficient evidence with which to draw definitive conclusions about what extent of HTFM is required to prevent local recurrence.

There is also confounding evidence that suggests tumour recurrence is not inevitable even when tumour cells are visible at the resection margins on histology. In studies where data on recurrence for canine STS with a positive HTFM has been reported, recurrence rates have ranged from as low as 17% and up to 100%;[9, 10, 23, 57, 76, 78, 86] across all studies, the mean rate of recurrence for an incompletely resected STS was 33% (38 of 114). Data from

these same studies reveals that STS can also recur even when the histological margins indicate complete resection has been achieved. Rates of recurrence in this instance can range from 0% up to 50%; across all studies, the mean rate of recurrence for a STS that was considered to have been completely resected on histologic examination was 10% (16 of 164).[9, 10, 23, 57, 76, 78, 86, 87] A recent meta-analysis determined that having a HTFM of >0mm reduced the risk of recurrence by approximately 60%.[79]

The inconsistencies between margin analysis and recurrence risk are not limited to canine STS; they have also been reported in human STS as well as many other neoplastic conditions.[88-95] Reasons for this inconsistency could be due to the inherent limitations of histological analysis that were described above. However, there are several tumour-related reasons that could explain why established histologic methods are unable to distinguish the STS that may have a higher risk of recurrence, irrespective of margin status. These reasons include the profile of the pseudocapsule, the presence of satellite nodules and the influence of the tumour microenvironment.

2.10.9 Effect of the pseudocaspule and microscopic invasion

One of the defining features of a STS is the pseudocapsule that surrounds the gross boundary of the tumour and creates a discernible circumscription to the tumour.[36] The pseudocapsule is formed initially by the compression and atrophy of the surrounding tissue as the tumour expands centrifugally. With continued expansion of the tumour, a reaction can develop between the capsule and normal tissue, which includes mesenchymal cell proliferation, an influx of inflammatory cells, haemorrhage, tissue oedema, and

angiogenesis.[36, 96] This area is termed the reactive zone and may sometimes be visible grossly as a discoloured area that surrounds the tumour.

Historically, the pseudocapsule has not been considered to be a barrier to tumour invasion and dissection through this cleavage plane – equivalent to a marginal resection – would lead to high rates of local recurrence.[5, 36] However, as discussed above, it is now recognised that some STS can be successfully managed with marginal resection margins.[9] Other STS – particularly those of higher grade – may require a wider HTFM. The extent of HTFM required to achieve adequate local control is therefore not binary and may vary according to individual characteristics of the tumour contour, and the microscopic invasion of cells beyond the gross boundary of the STS.

The peripheral contour of human STS has been described as either "pushing" (if no evidence of infiltrative growth was seen) or "infiltrative" (if the tumour contour was poorly defined, or satellite nodules were present).[97] A pushing growth pattern was more commonly observed with low-grade tumours, but a proportion (18%) of high-grade tumours can also display this characteristic. None of the tumours with a pushing growth pattern recurred after resection regardless of histologic margin, whereas local recurrence developed in 6 of 26 (23%) people after marginal excision and 13 of 56 (23%) people after wide excision in STS with an infiltrative growth pattern. In a similar study, an almost 7-fold increase (HR = 6.7, p=0.005; 95% CI 1.82-26.13) in local recurrence was seen in STS that had an infiltrative contour.[98]

There are four retrospective studies of canine STS that make an attempt to describe the contour of the STS.[38, 76, 78, 86] Each paper used a different criteria to describe whether the STS had a contour that was considered more or less invasive or infiltrative, so they are not directly comparable. One study showed that tumours with an infiltrative pattern were almost three times more likely to relapse (HR 2.45, 95% CI 0.61-9.89), but this finding was not significant. However, a significant relationship between a shorter recurrence free interval and dogs with an infiltrative tumour contour was demonstrated in another study.[86] In a further study, no recurrence was seen in 19 of 19 dogs with STS that were considered to have a less invasive/pushing growth pattern.[76]

The histological descriptions of human STS have also revealed discrete microscopic clusters of neoplastic cells – satellites or skip metastases - that extend some distance from the pseudocapsular boundary. These microscopic clusters, or even individual cells, are separated from the pseudocapsule by microscopically normal tissue. In one study of human STS, microscopic tumour nodules have been identified between 1cm and 4cm from the main mass in 30% of cases.[99] Satellite nodules are more commonly observed with high grade than low grade lesions. When low grade STS do develop satellite nodules, they tend to be clustered close to the periphery of the pseudocapsule.[36, 37] The microscopic diffusion of tumour cells about the circumference for both mast cell tumour and STS has been described in the dog in only two studies.[78, 100] In one study, satellite lesions were described if there was at least 1mm of microscopically non-neoplastic tissue interposed between the satellite lesion and the neoplastic cells that remained

in contact with the main tumour bulk. In this study of 19 STS, satellite lesions were observed in 6% of tumours with a mean distance of 3.8mm (range 2.9 – 17mm).[100] Almost 70% of the tumours in this study were low grade, and no high grade tumours were included. This may explain the relatively low incidence and distribution of the satellite lesions in this study, compared to what has been described in human STS.[99] In another study, satellite lesions were reported in 11 of 56 STS; these dogs had more than a 3.5 increased risk of relapse (HR 3.68 95% CI 0.81 – 16.69) when compared to 28 of 56 STS with an expansile profile, but this difference was not significant (p=0.09).[78] This study did not correlate the tumour profile with the grade of STS.

Taken overall, this evidence suggests the pseudocapsule of the STS is actually a more complex and nebulous structure than originally presumed and likely plays an important role in influencing recurrence of a tumour after surgery. In some tumours, the fibrous pseudocapsule may actually provide an effective barrier against tumour growth and infiltration but this probably holds true for a proportion of (mostly) low-grade lesions only.[36, 96, 101] In those instances, successful local control could indeed be achieved with excision of the mass including a narrow rim of normal tissue, as has been suggested by some authors.[23, 24] However, in other tumours, the reactive zone that surrounds the pseudocapsule is an area of nascent and evolving neoplastic activity, with isolated clusters of neoplastic cells and a permissive stromal microenvironment that supports cancer initiation, neovascularization and tumour migration. In these cases, there is a higher likelihood for tumour recurrence if the plane of surgical excision passes

through this area.[99, 102] The description of isolated tumour nodules located several centimeters from the tumour pseudocapsule may also provide an explanation for why local recurrence could still occur following complete removal of the gross tumour;[36, 99, 103] there are likely complex mechanisms at play in the tumour microenvironment that impact on which tumours recur, and those that do not.[104-106]

The influence of these differing tumour contours and extent of microscopic invasion of tumour cells into the surrounding tissues will likely have a profound, but as yet unmeasured impact on tumour recurrence. Because the presence of these characteristics cannot be reliably predicted for each individual tumour, there is an argument that wide surgical margins should be the appropriate strategy, as this would ensure that if satellite nodules are diffusely present around a particular STS they will be contained within the resected block of tissue.[1, 3, 5, 11] However, if it was that simple, existing data should demonstrate improved outcomes with increasing resection margins; as outlined above, the current literature does not support this correlation. This may suggest there are more complex elements involved. In human STS, the issue of 'how much to resect' has been circumvented by the routine inclusion of radiotherapy (adjuvant or neoadjuvant) into almost all treatment strategies.[44, 107-109] Radiotherapy in combination with surgery has allowed shrinkage of the resection margins without compromise for patient outcome.[42, 107] In the canine patient, routine inclusion of adjuvant RT is unlikely to become the standard of care for the treatment of STS, due to the costs and logistical challenges of delivering this treatment. Therefore,

efforts to develop novel prognostic markers or adjuvant treatments that are targeted to STS behaviour would assist efforts to manage this tumour.

2.11 Conclusion

STS is a complex disease and many uncertainties surround the biology of the tumour and the best options for clinical management. Historically, the tendency has been to recommend wide excision margins in all patients, but this is not fully supported by recent evidence. Nevertheless, it is accepted that inappropriately conservative treatment will affect the outcome for a patient with more aggressive disease.

The "biologic aggressiveness of a soft tissue sarcoma" was recognised in 1981 as the key factor in human STS to guide the selection of an appropriate surgical margin required to achieve local control.[36] Despite this awareness, the veterinary profession continues to struggle with the management of canine STS almost half a century later. Because there are no diagnostic tests that can reliably predict the amount of surgical margin required for a particular tumour, there is a mismatch between treatment and disease: some dogs are overtreated for their disease, resulting in large wound reconstructions or amputation when smaller surgical margins would have been effective. Other dogs are undertreated and suffer tumour recurrence and premature death due to inadequate initial treatment. Current evidence suggests it is not the extent of resection that influences successful patient outcome, but the biological behaviour of the tumour.[110] However, considerable deficiencies exist in the literature to help reliably determine the

prognosis for an individual patient. This highlights the need for more reliable and objective prognostic markers to be developed in canine and human STS.[13, 43, 72, 73, 109, 111-115] If prognostic markers for tumours with either favourable or aggressive behaviour could be identified and predicted with more confidence, more appropriate and targeted treatment could be provided.

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Chapter 3:

Retrospective study into prognostic influences on outcomes of 350 dogs treated with soft tissue sarcoma in primary veterinary care

3.1 Introduction

As discussed in the literature review, much of the current evidence for the behaviour of soft tissue sarcoma (STS) in dogs has been derived from cases managed in referral practice.[1-8] As a result, there is currently little known about the expected behaviour of a STS on a dog presented to a first opinion practice. It is suspected that first opinion veterinarians are more likely to refer a dog to a surgical specialist if it has a large STS or if it is located in a challenging location, but will continue to manage small, discrete and readily removal tumours themselves. Because scientific analysis of tumour management is mostly performed by specialists working in referral practice, it follows that most publications detailing the clinical behaviour of STS will be biased towards a more aggressive tumour subtype than those seen in first opinion practices.[3] This suggests that it is possible the currently recommended treatment guidelines are skewed and may not be appropriate for the STS managed in first opinion practices.

In a small pilot study that was performed prior to this PhD study, the outcome of 104 dogs with a diagnosis of STS that were managed in first opinion practice was studied.[3] In that study, only one in five dogs died as a result of tumour recurrence, which is fewer than would have been expected given that less than 10% of patients received treatment with wide surgical margins. Interestingly, resection margins were not prognostic for survival or tumour recurrence in that study. The results supported the hypothesis that STS managed in first opinion practice may have a less aggressive biologic behaviour, but also raised additional questions about the role of resection margins in the management of this disease. Further investigation, using a larger number of dogs and a broader range of STS subtypes, was required.

There were three aims of the study described in this chapter. Firstly, to determine the outcome for a larger cohort of dogs diagnosed with STS that were managed exclusively in first opinion practice. It was hypothesised that the recurrence rates and patient survival in this cohort would be better than the rates previously reported from studies of dogs with STS that were treated at referral clinics. The second aim was to evaluate clinical and histological features of the STS to determine if any of these features were correlated with differences in survival times or an increased tendency for local recurrence. The identification of such features may enable clinicians to be able to better predict the likely behaviour of a STS. The third aim was to establish an archive of histological tissue from these patients that could be used for novel biomarker discovery, as will be described in later chapters of this thesis.

3.2 Materials and Methods

Data for all canine STS that had been examined by a large, commercial diagnostic pathology service in the United Kingdom (Abbey Veterinary Services) during 2003 were retrieved from their database. Cases were included if the tumour was a primary occurrence of a STS, adequate clinical notes were available for review, patient outcome could be determined by questionnaire or telephone contact with the submitting veterinarian, and tissue blocks were available for histological review. A minimum follow-up period of at least 3 months after surgery was also required. Cases were excluded if the sample was found to represent an incisional biopsy only taken for diagnostic purposes.

A questionnaire that had been previously validated was sent to all veterinarians (Appendix 1).[3] Follow-up information requested from the veterinarian included the tumour size and location, any pre-operative evaluations performed, the extent of surgery undertaken, and the current status of the patient including dates of local recurrence, metastasis or death. The extent of resection margins obtained around the tumour were subdivided into four recognised subcategories of oncologic resection: **Marginal,** where the tumour was excised immediately about the pseudocapsule; **Local**, where the tumour was excised along with a margin of normal tissue that was less than 3cm wide; **Wide**, where the tumour was excised along with a margin of normal tissue that was greater than 3cm wide; and **Radical**, where the tumour was excised along with an entire body part such as a digit, tail or limb amputation.

Follow-up time was defined as the time from the date of surgery to the date of last follow-up or death. Local recurrence was defined as regrowth at the surgical site and metastasis was defined as occurrence of a STS at an anatomic location different from that of the initial surgery.

The disease-free interval (DFI) was defined as the time between the date of surgery to the date of local or distant tumour development or metastasis, whichever occurred first. Dogs were censored if they were lost to follow-up or were reported to have died from causes unrelated to the STS.

Soft tissue sarcomas were sub-classified into peripheral nerve sheath tumour (PNST), fibrosarcoma, giant cell tumour, perivascular wall tumour (PWT), myxosarcoma, or liposarcoma using light microscopy based on previously published criteria.[4] Immunohistochemistry was not used to assist with the differentiation of different STS subtypes. STS were also graded using criteria that have been described previously in the literature review (Table 3.1).

Due to difficulties in orientating neoplasms or because the veterinarian had only submitted parts of the overall STS, it was not considered possible to confidently assess the completeness of surgical excision by histology.

3.2.1 Statistical Analysis

All statistical analyses were performed with R (R version 2.8.1, R Foundation for Statistical Computing, Vienna, Austria). Deaths from tumour, local recurrence or metastasis were defined end points for the study. Any cases with an unknown finding within the category being analysed were not included in the statistical evaluation of that characteristic. The Kaplan-Meier

method was used to compare survival times according to age, sex, neuter status, clinical signs, duration of signs, tumour size, tumour type, histological

Table 3.1:
Grading System for Cutaneous and Subcutaneous STS in the Dog (modified after Dennis MM, McSporran KD, Bacon NJ, et al.: Prognostic factors for cutaneous and subcutaneous STS in dogs. Vet Pathol 48:73-84, 2011)[4]

Score Criteria	Score = 1	Score = 2	Score = 3
Differentiation	**Well-differentiated:** Sarcomas most closely resembling normal adult mesenchymal tissue, by type	Moderately well-differentiated: Sarcomas for which histologic type can be determined, although differentiation is poor	**Poorly differentiated:** Undifferentiated sarcomas, sarcomas of unknown type
Mitotic score:	0-9 mitoses per 10 hpf	10-19 mitoses per 10 hpf	>19 mitoses per 10 hpf
Necrosis score	No Necrosis	≤50% necrosis	>50% necrosis

***Histologic grade** = Sum of (differentiation score + mitotic score + necrosis score)*
Grade I score = 3 Grade II score = 4 or 5 Grade III score = 6

characteristics (i.e. differentiation, necrosis, mitotic score, grade), and the development of local or distant tumour recurrence. A value of $p<0.05$ was considered significant. Prognostic factors that on univariate analysis had a value of $p<0.1$ were included in a multi-variable analysis using Cox's proportional hazards model to help evaluate their independent influence on outcome. Backward selection methods were used to create a fixed effects model, retaining only those values that had a p value of <0.05. The assumption of proportional hazards was assessed by plotting the Schoenfeld residuals as a function of time.[9]

Finally, logistic regression analysis was performed to identify categories of significance between patients whose tumours recurred within the first 365 days (early recurrence) and a second group of patients whose tumours did not recur for more than 2 years after surgery (late recurrence).

3.3 Results

3.3.1 Cases included in the study

A total of 1144 questionnaires were sent out; 632 were returned, 88 of which were incomplete, resulting in a 47.5% return rate. A further 67 cases were excluded from further analysis for any of the following reasons: an inadequate follow-up interval or the tumour was a recurrence from a previous surgery. A total of 477 cases remained for histological review.

Tissue blocks were available for all 477 cases; however, 37 of these were excluded because the tissue sections produced from them were of poor quality and this poor quality prevented critical evaluation. A further 90 tumours were discarded after histological review, as they were not considered to be consistent with STS on histology. This left 350 cases for final analysis.

3.3.2 Demographics

Of the 350 dogs included in the study, 195 were female and 155 were male; 54% of the females and 34% of males were neutered. The median age at time of diagnosis was 10 years, with a range of 3 to 16 years. There were over 50 different breeds reported, with the 4 most common being crossbreed (78

[22%]), Border collie (44 [13%]), Labrador retriever (34 [10%]), and boxer (26 [7%]) (Table 3.2).

Table 3.2:
Summary of soft tissue sarcomas included in the archive

Characteristic	Groups	Number
Age	<=8 years >8 years	93 251
Sex	Male Female	155 195
Location	Head Limb Trunk	16 211 123
Size	<1 cm 1-5cm >5cm	13 142 68
Palpable	Discrete Firmly Attached	106 128
Diagnosis	Fibrosarcoma Giant cell tumour Liposarcoma Myxosarcoma Peripheral nerve sheath tumour Perivascular wall tumour	66 1 6 13 242 22
Degree of Resection	Marginal Local (<3cm) Wide (>3cm Amputation	143 117 13 19
Metastasis	No Yes	
Grade	Grade 1 Grade 2 Grade 3	231 95 22
Differentiation	Well-differentiated Mod. well-differentiated Poorly differentiated	170 153 25
Mitotic rate	0-9 mitoses/10hpf 10-19 mitoses/10hpf <20 mitoses/10hpf	274 50 24
Time to Recurrence	Early (<365 days) Late (>730 days)	37 13
Local recurrence	No Yes	260 73
Died due to sarcoma	No Yes	238 58

3.3.3 Tumour details

Soft tissue sarcoma were located on the limbs in 211 (60%) cases, the trunk (including the tail (6) and perineal (3) area) in 123 (35%) cases and the head in 16 (5%) cases.

Thirteen (4%) of the STS were reported to be less than 1cm in size, with 142 tumours (41%) sized between 1-5cm and 68 (19%) being larger than 5cm in diameter. The size of 127 STS (36%) was not recorded in the clinical notes.

The STS were described by the submitting veterinarians as mobile and discrete in 106 (30%) cases but were considered fixed to the surrounding tissues in 128 (37%) cases. In 116 (33%) cases, the veterinarians were unable to recall the gross nature of the tumour on palpation.

3.3.4 Tumour management

In 229 (65%) cases, no pre-operative investigations were performed prior to mass removal. Fine needle aspiration (FNA) of the mass was performed in 75 cases (21%), with results providing confirmation or strong suspicion for a STS or spindle cell neoplasm in 59 cases (17%). Some respondents commented that FNA was principally used to rule out conditions such as lipoma or mast cell tumour and samples were not always submitted to an external laboratory for analysis. Incisional biopsy and histological examination had been performed prior to surgery in 15 (4%) cases.

Clinical staging of tumours was infrequently performed. Pre-operative haematology and biochemical evaluations were performed in 16 (4%) cases.

Evaluation of the draining lymph node by FNA was performed in just 1 case. Thoracic radiographs were performed in 16 (4%) cases. Abdominal ultrasound was not performed in any case.

The extent of surgical resection was described as marginal in 143 (41%) cases and local in 117 (33%) cases. Wide resections of 3cm or more about the tumour were performed in just 19 (5%) cases, with radical resections (amputation, which included 4 toe and 2 tail amputations) performed in 13 (4%) cases. The extent of resection could not be recalled by the veterinarians in 58 (17%) cases. No information was available on the deep margins in any case. If a neoplastic condition was suspected from pre-operative biopsy or cytology, there was a tendency for a wider surgical excision to be performed ($p=0.007$).

3.3.5 Histological analysis

Tumours of presumed peripheral nerve origin (i.e. PNST) were most common (242 of 350, 70%). Fibrosarcoma were the next most common (66 of 350, 18.8%) followed by PWT (22 of 350, 6%). Myxosarcoma, liposarcoma and giant cell tumour were diagnosed less commonly.

There were 231 (66%) grade 1 tumours, 95 (27%) grade 2 and 22 (6%) grade 3 tumours. Necrosis was absent in 242 (70%) cases, present and representing less than 50% of the tumour in 94 (27%), and present and representing more than 50% in 12 (3%) cases. The mitotic rate was distributed as 0-9 per 10 high power fields (hpfs) (274, 78%), 10-19 per 10 hpfs (50, 14%) and >20 per 10 hpfs (24, 7%). Tumour differentiation was classified as well-differentiated

in 170 STS (49%), moderately well-differentiated in 153 (44%) and poorly differentiated in 25 (7%).

3.3.6 Clinical Outcomes

Follow-up times ranged from 102 to 2192 days, with a median follow-up time of 785 days. Over 85% of the study population had a follow-up time of more than 12 months, with 35% of cases being followed for longer than 3.5 years (1290 days) (Table 3.3).

From Kaplan Meier analysis, the overall mean survival time was 1796 days, equivalent to almost 5 years following surgery (Figure 3.1). The median survival time for all dogs was not reached; estimated 1-, 2-, and 5-year survival probabilities were 94%, 86% and 70%, respectively.

During the study period, 277 dogs died, representing almost 80% of the study population. Death was attributed to the STS in 58 cases (16.5%). A reason for death or euthanasia was not recorded in 54 cases (15.4%). In 16 of these cases, local recurrence or metastasis had been documented, so death due to the STS was a possibility.

Table 3.3:
Distribution of tumour types and outcome

Tumour Type	Frequency	Local Recurrence	Metastasis	Tumour related death	Mean Survival Time and Range (days)
Peripheral nerve sheath tumour	242 (69.1%)	51 (21.1%)	27 (11.1%)	39 (16.1%)	1804 (102-2192)
Fibrosarcoma	66 (18.8%)	14 (21%)	11 (16.7%)	16 (24.2%)	1499 (109-1997)
Perivascular wall tumour	22 (6.3%)	2 (9.1%)	0	0	894 (181-2020)
Myxosarcoma	13 (3.7%)	4 (30.8%)	1 (7.7%)	0	1027 (221-2006)
Liposarcoma	6 (1.7%)	1 (16.7%)	1 (16.7%)	2 (33.3%)	1444 (221-2012)
Giant cell tumour	1 (0.3%)	1 (100%)	0	1 (100%)	1818
Total	350	73 (20.9%)	40 (11.4%)	58 (16.6%)	1797

Figure 3.1:
Kaplan Meier plots showing survival outcome for all dogs with a soft tissue sarcoma

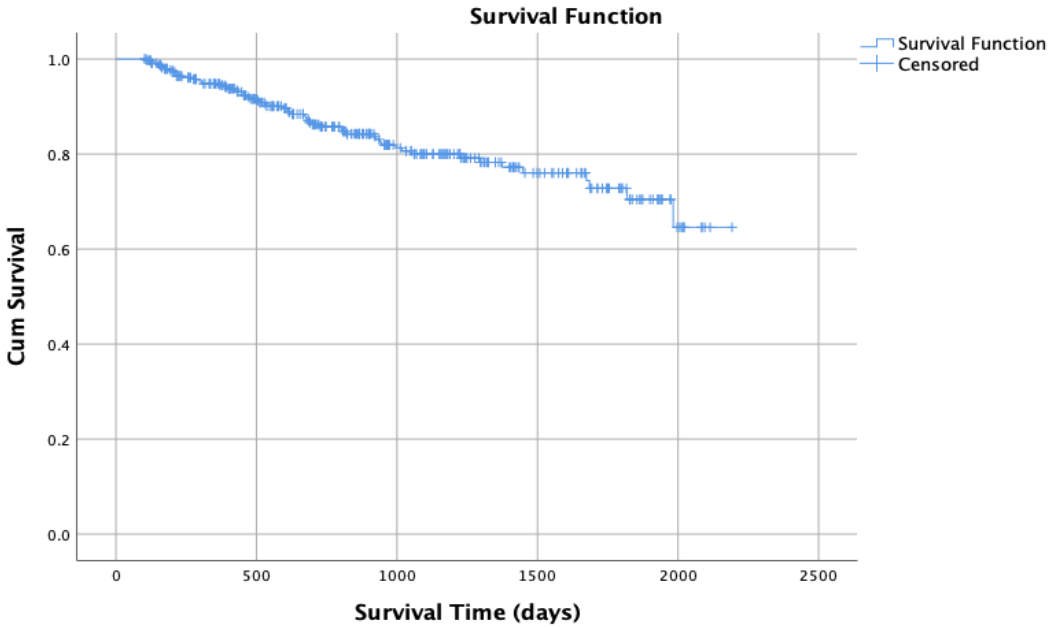
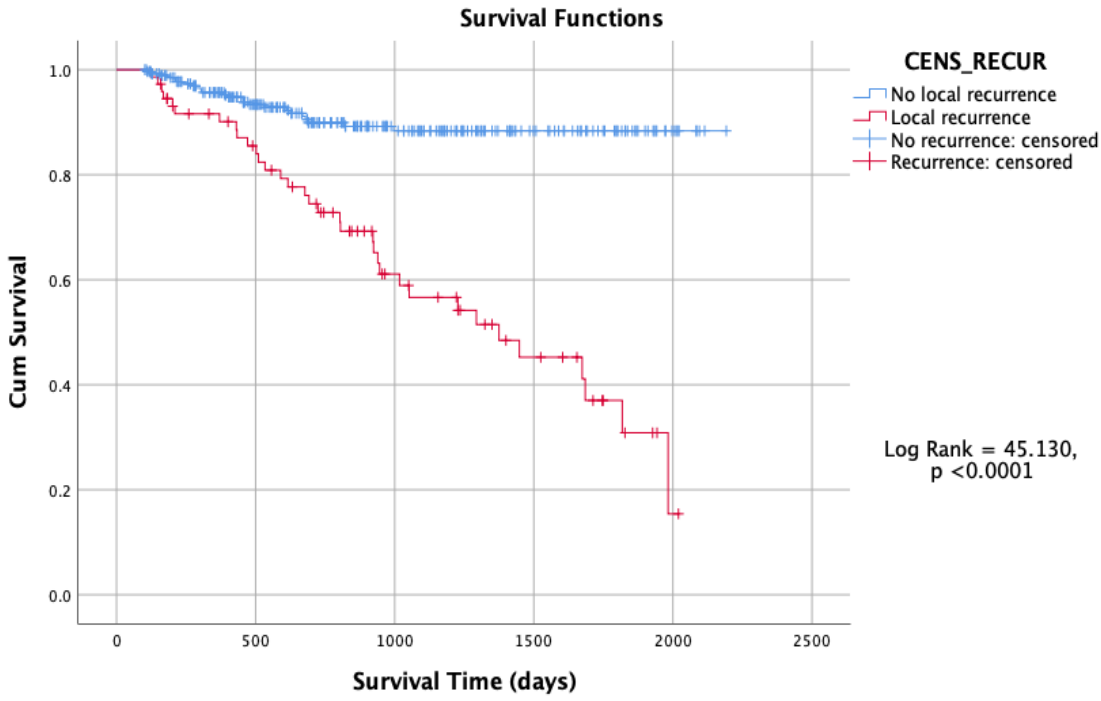


Figure 3.2:
Kaplan Meier plots for survival time, where euthanasia was performed due to local recurrence of the tumour



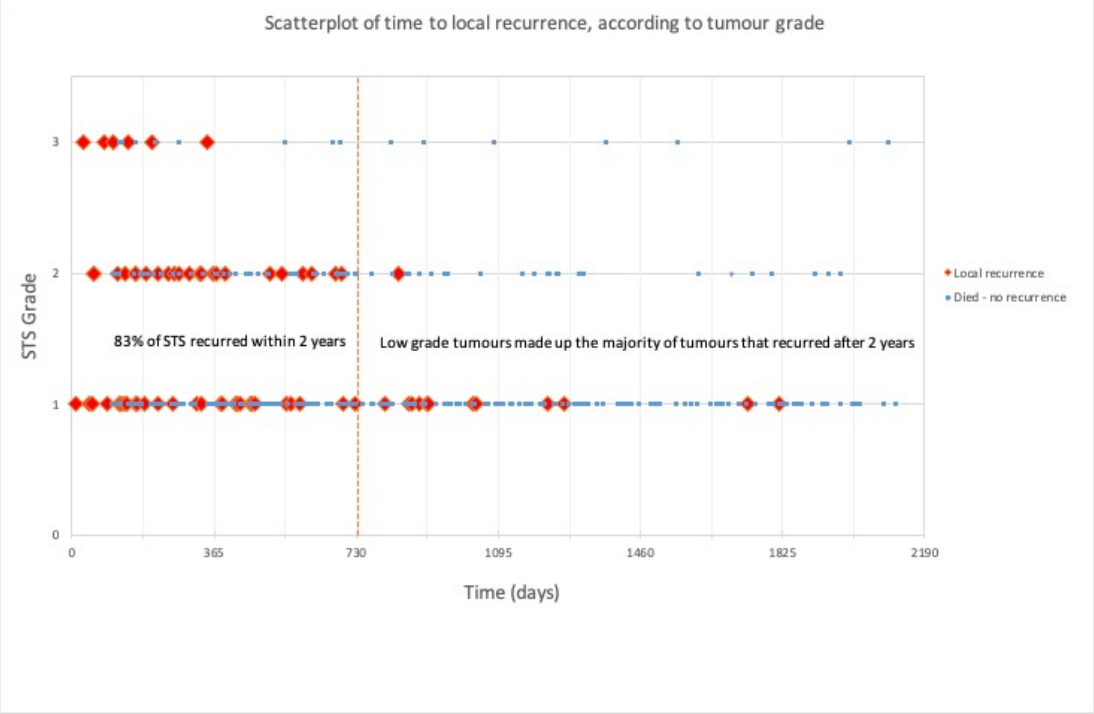
Local tumour recurrence occurred in 73 (20.8%) cases and did not occur in 260 (74.2%) dogs (Figure 3.2). In 17 dogs (4.8%), the submitting veterinarian was unable to confirm whether recurrence had occurred or not. The median DFI was not reached. The mean DFI for all dogs was 637 days, with estimated 1-, 2-, and 5-year disease-free probabilities being 89%, 78%, 66%, respectively.

When all 73 dogs with STS recurrence were considered, recurrence was observed within 365 days of surgery in 37 (51%) dogs, with an additional 23 (32%) dogs having tumours recurring between 365 days and 2 years after surgery. Overall, tumour recurrence was observed for 60 of 73 (83%) dogs within 2 years of surgery. Recurrence was observed between 2 and 4 years

after surgery in 11 of 73 (15%) dogs and more than four years after the original surgery in 2 of 73 (3%) dogs.

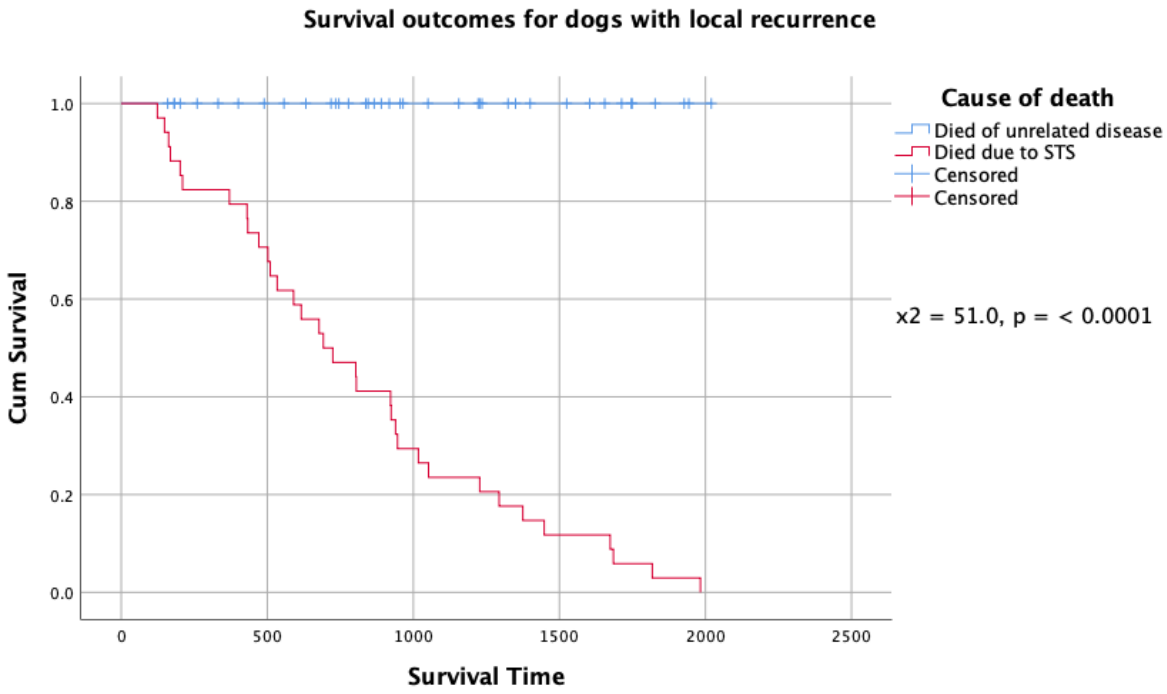
Tumour grade was significantly associated with a risk of tumour recurrence (p = 0.0001). Low grade STS were significantly more likely to recur more than 2 years after surgery compared to medium or high grade tumours (p=0.03) (Figure 3.3). Additionally, STS that had a mitotic rate of less than 10 per 10 hpfs were significantly more likely to recur more than 2 years after surgery than STS that had a mitotic rate of greater than 10 per 10hpfs (p=0.03).

Figure 3.3:
Low grade tumours were significantly more likely to recur more than 2 years (730 days) after surgery compared to more higher grade tumours



In the 73 dogs that had a STS recur at the original surgical site after excision, 34 dogs were euthanised due to the presence of a sarcoma while 39 dogs apparently died due to causes unrelated to the recurrence of the sarcoma. Dogs that were euthanised because of a recurrent STS had a median post-recurrence survival time of 708 days (range 124-1983 days) which was significantly shorter than the mean post-recurrence survival time of 1103 days (range 159-2020 days) for dogs that died due to unrelated causes while a recurrent STS was present (log rank 51.0, p = <0.0001) (Figure 3.4).

Figure 3.4:
Death due to tumour recurrence was not inevitable. In this study, 16 dogs that developed local recurrence died from unrelated causes, with a mean survival time of 1008 days.



Metastatic disease was reported to have developed in 40 (11%) dogs with STS. The median metastasis-free interval for these cases was 550 days (95% CI

381-718 days). Metastases were reported for all types of STS except PWT. Metastasis was reported in 8 of 14 grade 3 tumours (36%), 13 of 95 grade 2 (14%) and 19 of 231 grade 1 tumours (8%). Metastasis was significantly more likely to occur in grade 3 than grade 1 STS ($\chi^2 = 16.7$, $p < 0.0001$). Sites of suspected metastasis included subcutaneous sites elsewhere on the body (9 of 40), spleen (4 of 40), lungs (10 of 40), liver (5 of 40), central nervous system (CNS) (1 of 40), pelvis (1 of 40), bowel (1 of 40) and nasal chamber (1 of 40), or combinations of these sites (4 of 40). The site of metastasis was not recorded in 4 of 40 cases. A diagnosis of sarcoma metastasis was determined by either imaging alone (21 of 40) or histology alone (4 of 40). In 12 cases, the veterinarians did not indicate how a diagnosis of metastasis was confirmed. There was no correlation between the development of local recurrence and metastasis ($\chi^2 = 1.48$, $p = 0.2$).

3.3.7 Analysis of prognostic features influencing survival

A number of individual clinical characteristics were analysed to show their relationship to long term patient survival after surgery. Patient age, tumour size, the palpable characteristics of the tumour, tumour type, grade, mitotic rate, percentage tumour necrosis and recurrence of the tumour were all found to have a significant influence on survival. Only patient sex and neuter status were not found to influence survival. The details of this analysis are presented in more detail below, with Kaplan-Meier graphs for each characteristic presented in Figure 3.5:

Age: Of the 66 dogs with a STS that were less than 8 years of age at diagnosis, only 9 (14%) dogs died due to neoplasia. This rate was significantly lower than dogs that were greater than 8 years of age at diagnosis, of which 48 of 278 (17%) dogs died due to STS ($\chi^2 = 6.1$, p=0.01). Dogs that were 8 years of age or older had a more than double increased rate of death from their tumour compared to dogs less than 8 years of age (HR 2.25, p = 0.016, 95% CI 1.2 – 4.3).

Size: Dogs with a STS that was smaller than 1cm in size at the time of surgery were significantly less likely to die (1 of 13 dogs died, 8%) from their tumour compared to dogs with a tumour greater than 5cm (19 of 68, 28%) ($\chi^2 = 9.6$, p=0.002).

Palpable characteristics: STS that were discrete and mobile within the tissues were significantly less likely to cause the death of the dog (8 of 106, 7.5%), compared to tumours that were fixed and immobile (33 of 128, 25.8%) ($\chi^2 = 17.2$, p = 0.0002).

Grade: Only 31 of the 231 (13%) dogs with a low grade STS died as result of their tumour. By comparison, 9 of 22 (40.9%) dogs with a high grade tumour died from their STS. This difference was statistically significant ($\chi^2 = 17.6$, p=0.0002).

Differentiation: Seven out of 25 (28%) dogs with poorly differentiated STS died as a result of their tumour. This compared to 25 out of 170 (15%) and 26 out of 153 (17%) with well differentiated and moderately differentiated

tumours, respectively. This difference was not statistically significant (χ^2 = 4.2, p = 0.12).

Mitotic rate: Nine out of 24 (37.5%) dogs that had a STS with a mitotic rate of greater than 20 per 10 hpfs died as a result of their tumour. This compares with tumour-related death in 41 of 274 (15%) dogs with a mitotic rate of less than 10, and 8 of 50 (16%) dogs with a mitotic rate between 10 and 20. This difference was statistically significant (χ^2 = 11.8, p=0.003).

Necrosis: Half of the dogs (6 of 12) with a STS that was more than 50% necrotic on histological assessment died as a result of their tumour. This compared with just 14% (33 out of 209) for tumours with little or no necrosis, and 20% (19 out of 94) for tumours with <50% necrosis. This difference was statistically significant (χ^2 = 3.9, p=0.0001).

Tumour recurrence: Tumour recurrence was associated with more than a five-fold risk of death (HR 5.2, p<0.0001; 95% CI 3.1-9.0). However, some dogs did experience prolonged survival despite tumour recurrence. In five cases, tumours were reported to have recurred within two months of the original surgery, yet these dogs were recorded as dying for non-tumour related reasons from between 174 and 401 days after surgery.

Resection margins: Sarcoma-related death occurred in 24 of 143 (17%) dogs who underwent a marginal excision of their tumour, 14 of 117 (12%) dogs with local (<3cm) excision margins, 3 of 19 (16%) dogs with wide excision, and 4 of 13 (31%) with amputation. These differences were not statistically significant.

Sex: There were 195 female dogs, and 34 died (17.4%) as a result of their tumour. For male dogs, 24 of 155 dogs (15.4%) died from their tumour. This difference was not significant ($\chi^2 = 0.3$, p=0.6).

Neuter status: Fifteen of the 105 (14%) neutered females and 19 of the 90 (21%) females who remained intact, died from their tumour. Seven of 55 (13%) castrated dogs died from their tumour, while 17 of the 102 (17%) entire dogs died. Overall, of the 158 animals that had been neutered, tumour related death occurred in 22 (14%), while 36 of the 192 (19%) who remained entire died from their tumour. This difference was not significant ($\chi^2 = 0.3$, p=0.6).

When all prognostic factors with a significant influence on survival (to a value of p<0.1) were evaluated by multi-variable analysis, only the palpable characteristics and grade of the tumour were found to be significant. Tumours that were considered firmly attached to the underlying tissues were found to increase the likelihood of death by four times (HR 4.0, p=001; 95% CI 1.8-8.7). Compared to a dog with a grade 1 tumour, a dog with a grade 2 tumour was almost twice as likely to die from their STS (HR 1.9, p = 0.04, 95% CI 1.0 – 3.3), and one with a grade 3 tumour was more than 4 times more likely to die from tumour-related causes (HR 4.2, p = 0.0001, 95% CI 2.0 – 9.0).

The test of the proportional hazards assumption confirmed the model was a reasonable fit (p=0.13).

Figure 3.5:

Kaplan Meier survival curves for individual tumour characteristics that had a significant influence on patient survival after surgery

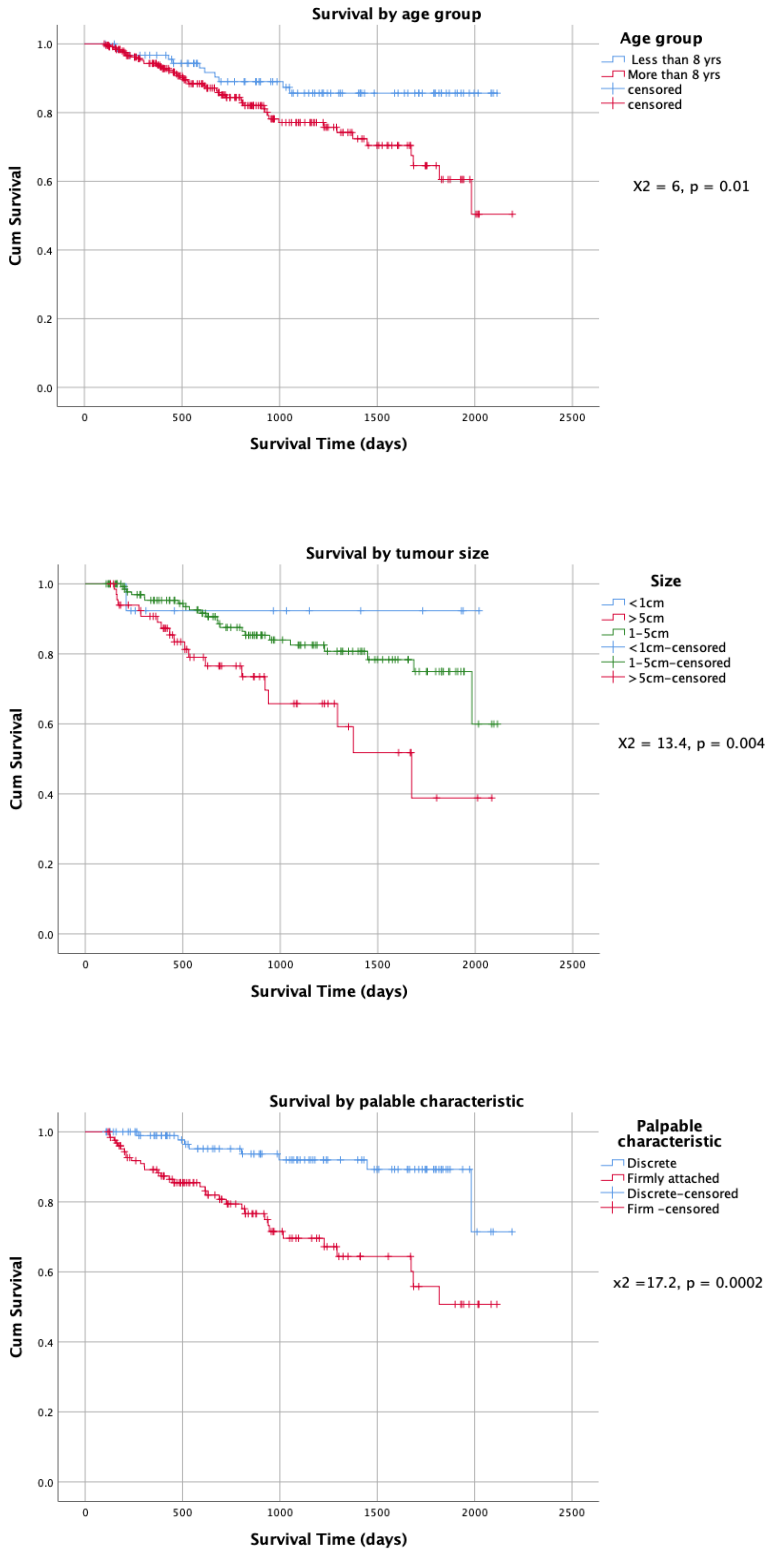
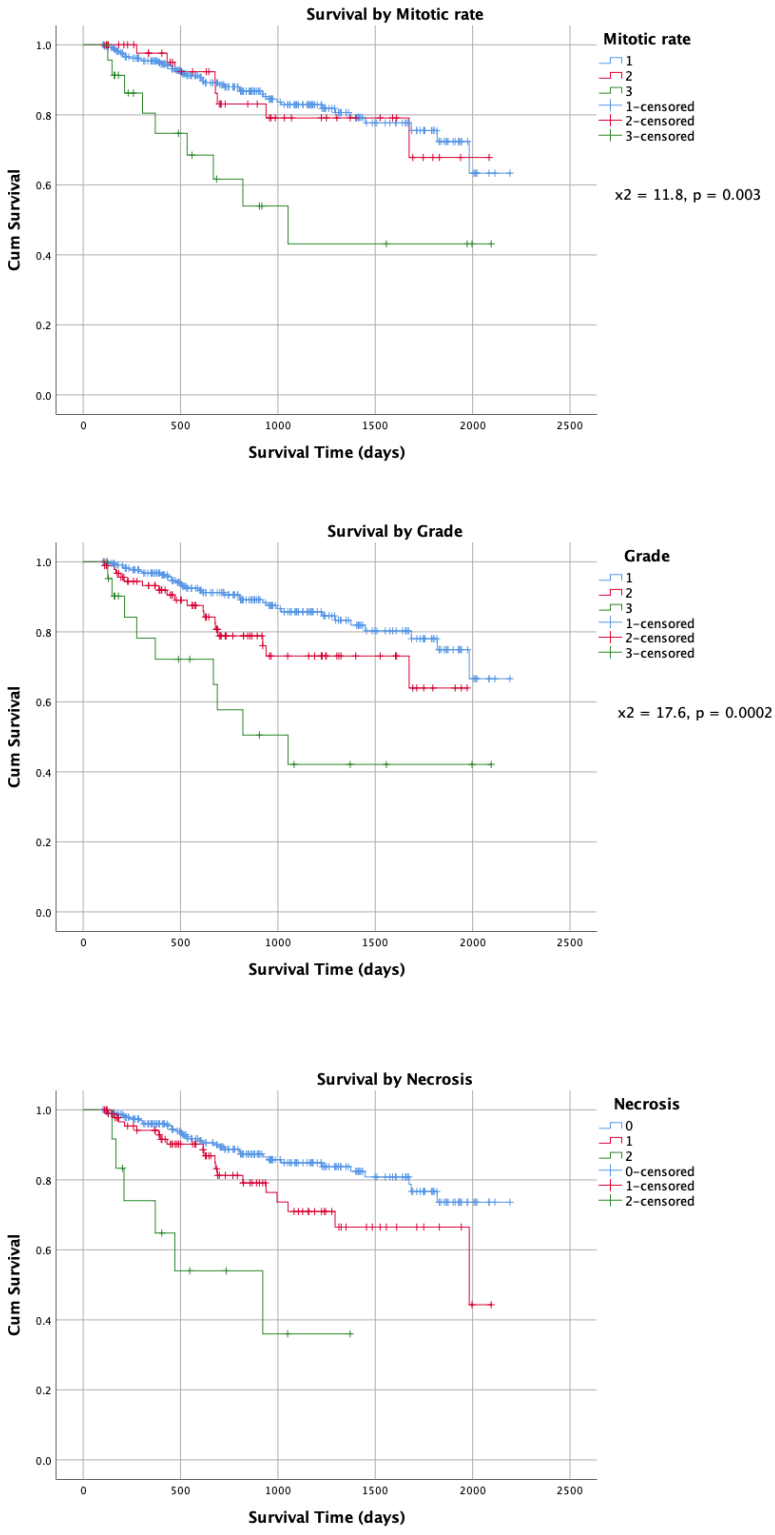


Figure 3.5 (continued):
Kaplan Meier survival curves for individual tumour characteristics that had a significant influence on patient survival after surgery



3.3.8 Analysis of prognostic features influencing local recurrence:

When the same individual clinical characteristics were analysed to show their relationship to local recurrence of the tumour after surgery, tumour grade, mitotic rate, necrosis and the histological diagnosis were found to have a significant association with the disease-free interval. The remaining characteristics studied were not significantly associated with tumour recurrence. The details of this analysis are presented in more detail below, with relevant Kaplan Meier curves illustrated in Figure 3.6.

Grade: Local recurrence of the STS occurred in 42 of 231 dogs (18.2%) with a low grade STS, compared to 6 of 22 (27.3%) dogs with high grade tumours. This difference was statistically significant ($\chi^2 = 6.8$, $p = 0.03$).

Mitotic rate: Eight out of 24 (33%) dogs that had a tumour with a mitotic rate of greater than 20 per 10 hpfs developed a local recurrence after surgery. This compares with a recurrence rate of 19% (52 of 274) dogs with a mitotic rate of less than 10, and 24% (12 of 50) for dogs with a mitotic rate between 10 and 20. This difference was statistically significant ($\chi^2 = 6.67$, $p=0.04$).

Necrosis: Eight of the twelve dogs (67%) with a STS that was more than 50% necrotic on histological assessment developed a local recurrence after surgery. This compared with just 16% (49 out of 242) for tumours with little or no necrosis, and 20% (15 out of 94) for tumours with <50% necrosis. This difference was statistically significant ($\chi^2 = 27.9$, $p<0.001$).

Differentiation: Seven out of 25 (28%) dogs with poorly differentiated STS developed local recurrence. This compared to 38 out of 170 (22%) and 27 out

of 153 (18%) with well differentiated and moderately differentiated tumours, respectively. This difference was not statistically significant ($\chi^2 = 2.7$, $p = 0.23$).

Histologic diagnosis: Recurrence was observed in 51 of 242 (21%) PNST, 4 of 12 (31%) myxosarcoma and 14 of 66 fibrosarcoma. Only 2 of 22 (10%) PWT developed local recurrence, but this difference was not significant.

Resection margins: Local recurrence developed in 37 of 143 (26%) dogs after marginal excision, 23 of 117 (20%) dogs after local (<3cm) excision, 2 of 19 (11%) dogs after wide excision, and none of the 13 (0%) dogs who underwent an amputation. Resection margins were not significantly associated with increased rates of recurrence.

Sex: There were 195 female dogs, and 38 (19%) developed local recurrence of their tumour. For male dogs, 35 of 155 dogs (23%) developed local recurrence. This difference was not significant.

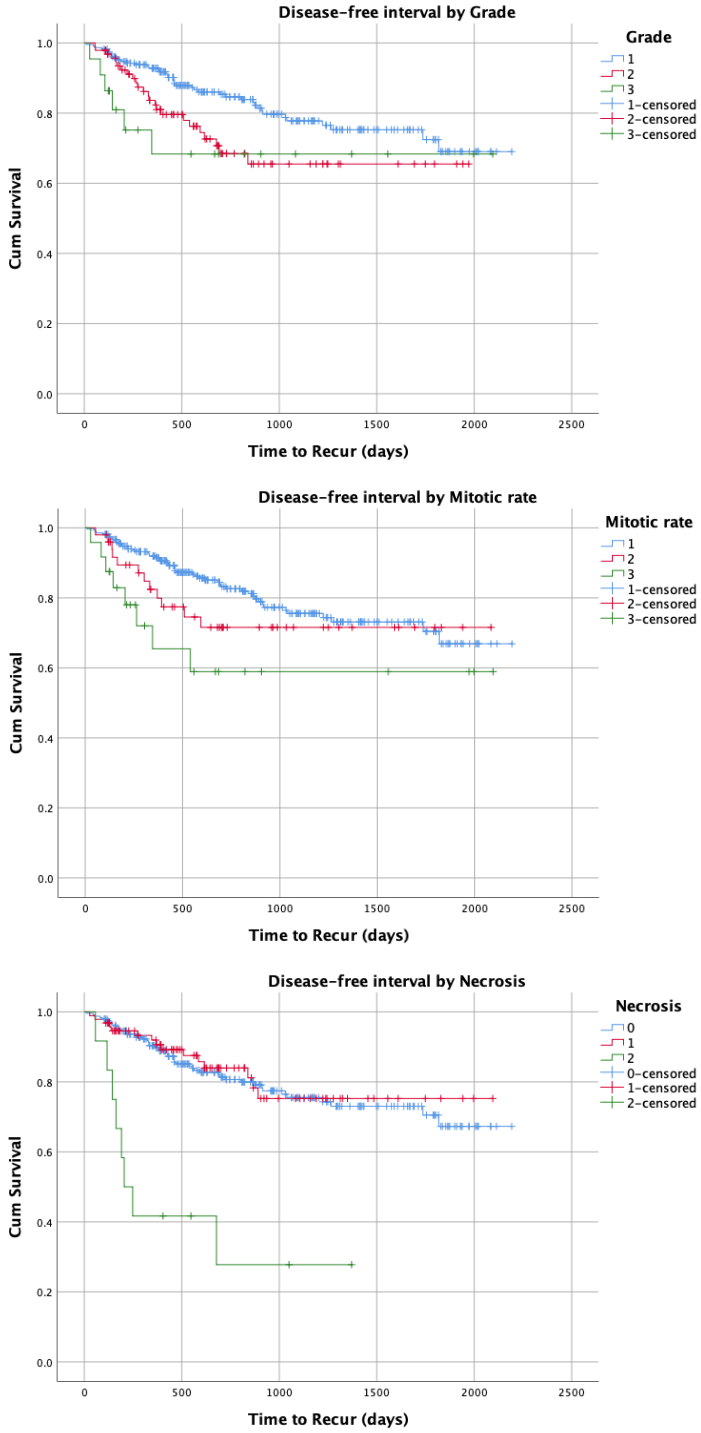
Neuter status: Twenty-three of the 105 (22%) spayed females, and 15 of the 90 (17%) females who remained intact, developed local tumour recurrence. Twelve of the 53 (23%) castrated dogs and 23 of the 102 (23%) entire dogs developed local recurrence. Overall, of the 158 animals that had been neutered, tumour recurrence occurred in 35 (22%), while 38 of the 192 (20%) who remained entire developed local recurrence. This difference was not significant ($\chi^2 = 0.3$, $p=0.6$).

On multivariable analysis, only tumour grade was significant for recurrence, with high grade tumours having an almost 6 times increased hazard for

recurrence compared to low grade tumours (HR 5.8 p<0.001; 95% CI 2.2-14.8).

Figure 3.6:

Kaplan Meier survival curves showing influence of individual tumour characteristics on tumour recurrence



3.4 Discussion

This study showed that only 16.5% of dogs with STS that were treated at first opinion veterinary practices died due to their cancer. By comparison, in a study of STS treated at a referral practice, a mortality rate of 33% was reported.[7] Therefore, the results of this study support the hypothesis that the outcome for canine STS in a general population of dogs may be better than what has previously been reported in the literature.

Although death is the definitive endpoint measure for a patient with cancer, preventing local regrowth of the tumour following surgical resection is perhaps the more important challenge in the management of STS. The current study has shown that recurrence is associated with a more than 5-fold increased risk of death, with STS recurring in around one out of five patients after surgery. This tendency for STS to recur after surgery was first reported by Bostock and Dye in 1980.[10] They reported recurrence rates of up to 60% following surgery for STS; due to this high rate of recurrence they recommended aggressive surgical resection to improve local control. When Kuntz and others (1997) showed that wide resection about the tumour reduced the recurrence rate to just 15%,[7] it became accepted that the appropriate margins for surgical resection of STS should comprise at least 3cm of tissue lateral to the tumour, and one fascial plane deep to the tumour, as used in their study. Most textbooks and review papers that detail the treatment of STS emphasise the importance of this more aggressive surgical strategy.[5, 6, 11, 12] Despite those recommendations, the current study has found that patients with STS operated in first opinion practice can have

similar or better outcomes than would have been predicted by the current literature despite only 5% of dogs receiving the wide resection margins recommended for effective control of STS. Recurrence was observed in 10% of dogs with wide margins and around a quarter of dogs that had a marginal excision, but this difference was not significant. On overall analysis, the current study suggests that the extent of surgical resection of the STS does not significantly influence the likelihood of tumour recurrence or overall survival.

As discussed in the literature review, other authors have challenged the notion that wide surgical excision margins are essential in all dogs with STS.[1-3, 13] In a prospective clinical study, 100% local disease control and 93% one-year disease-free interval was achieved in 14 dogs after removal of the tumour with just 1cm lateral margins and a single deep fascial plane.[1] Other authors have reported a 10% local recurrence rate for 35 dogs with spindle cell tumours of the extremities treated by marginal excision only.[13] In that study, no recurrence was observed in dogs where at least 3mm of normal tissue was found between the tumour and surgical margins on histological review. The results of the current study support these earlier studies and suggests that many STS do not recur after surgery, even when wide resection is not achieved.

At the start of this PhD, it was speculated that STS selected for management in first opinion practice may have a tendency for a less aggressive behaviour than those reported in the first clinical publication on this tumour type in 1980.[10] Since that study was published, our understanding of the

behaviour of soft tissue tumours has improved, together with the development of specialist services and the pet-owning public's acceptance of cancer treatment. These events raise the possibility that STS now selected for management in first opinion practice will have an improved outcome because: 1) owners may present their animals for treatment of a mass sooner than previously; 2) potentially aggressive masses (i.e. large masses, those that had demonstrated a period of recent rapid growth, were located in a difficult location, or had recurred following a previous resection) are now more likely to be referred for specialist management. Evidence for such a selection bias was evident in this study population, with obvious differences in the proportion of tumour grades when compared to historical publications derived from referral practice. Grade is recognised as an important indicator of tumour aggression, and is one of the most validated criteria to predict recurrence after surgery.[4] In the current study, two-thirds of the tumours were classified as low grade, while high grade lesions represented only 6.3% of the population. By comparison, in studies derived from a referral population of tumours, the proportion of high grade tumours is almost 3 times higher (i.e. between 17-29% of the sample population).[7, 14, 15] The trend for more low grade tumours to be excised in first-opinion practice is also supported by an analysis from pathology submissions to veterinary diagnostic laboratories in the United Kingdom where 87% of all canine STS submitted from first opinion practice over a three year period were classified as low grade, with considerably smaller numbers of intermediate (8%) and high grade (3%) tumours.[3]

The size of the STS managed in the current study also supports a selection bias when compared to other historical publications. Over two-thirds of the tumours in the current study were less than 5cm in size, with only 68 of 223 (30%) being recorded as more than 5 cm. Although direct comparisons are difficult due to differences in reporting methods, papers derived from a referral population of surgical patients have described median tumour sizes of 4.7cm, 5cm or 6cm.[1, 7, 10] Larger tumours are generally considered to have poorer prognosis;[4, 7, 16] this is supported by results of the current study where STS larger than 5cm were associated with a more than 5 times increased daily hazard for death when compared to tumours that were <1cm in size. Tumour size probably affects prognosis by influencing the ability to achieve complete resection.[11] The increased proportion of small tumour sizes in the current study may suggest that first opinion practitioners are more prepared to operate on smaller masses but will refer larger masses for treatment at specialist centres.

The impact of the bias identified in the current study is important. Most clinical studies on STS are performed at referral centres, so the prevailing literature represents a skewed population of tumours that are likely to be larger, located in challenging locations and have a more aggressive biological behaviour compared to the STS managed in first opinion practice. This bias alters our understanding of the true biology of STS within the general canine population and the treatment recommendations provided to veterinarians.

If surgical margins are not influential on outcome, it follows that there must be other factors that influence recurrence of a STS after surgery. In order to

help predict which STS are more likely to recur after excision, a number of clinical and histological features were evaluated for their use to predict tumour recurrence or death of the dog. Of the factors that were evaluated, the age of the patient, tumour size, the palpable characteristics of the tumour, grade and the histologic type were all found to have prognostic significance for either patient survival, tumour recurrence or both. Possible explanations for these findings are detailed below:

Age: The wide age range (3 – 16 years) for STS incidence in this study was similar to that reported in earlier studies.[7] In the current study, dogs less than 8 years of age were found to have a reduced chance of death from their tumour compared to dogs greater than 8 years old. Although significant, the actual difference was small (13.6% vs. 17.3%). A decision by an owner to euthanise their dog because of a recurrent STS may be influenced by many factors. In the older dog, for example, the presence of concurrent disease conditions such as arthritis, heart disease or other age-related disorders, may impact on an owner making an 'end of life' decision with criterion that would be different to those influencing the same decision in a younger dog.

Tumour size: In the current study, dogs with tumours larger than 5cm had a significantly poorer outcome than dogs with tumours that were only 1cm in diameter. These results support several previous studies of canine and human STS that reported that patients with STS that were larger than 5cm in diameter have shorter disease-free intervals and survival times than patients with STS that were smaller than 5 cm in diameter.[7, 16-22] However, this association has not been reported consistently and other authors have not

found any association between tumour size and outcome.[3, 10, 23] There are a number of reasons why a larger STS would be associated with a worse outcome. It is possible that larger tumours are more difficult to remove than smaller tumours, with surgeons being less willing or able to maintain their chosen resection boundaries about the entire circumference of the mass. Resection of a large STS requires a detailed anatomic knowledge of the affected tissues, combined with a sound understanding of oncologic principles, surgical skills and peri-operative nursing care. It is possible that while an inexperienced surgeon can maintain a measured cushion of normal tissue about the tumour during the initial stages of a dissection, they become less confident once the surgery extends deeper and wider into the body. There may be a tendency for the dissection to stray into the relative safety of the cleavage plane that surrounds the pseudocapsule of the tumour, which increases the risk for microscopic deposits of tumour tissue to be left behind.[24] One study has reported a significant increase in the risk of an incomplete excision when the tumour was excised by a surgical resident compared with a specialist surgeon.[16] Similar variances are identified in human medicine, prompting demands for STS to only be operated by trained surgeons in dedicated centres.[25, 26] It is also possible that STS that have attained a large size have done so because they have grown more rapidly and therefore have a more aggressive biological behaviour. Some authors have suggested that a history of sudden or rapid growth by the STS, or the presence of gross tumour necrosis and ulceration within the tumour, may imply a more aggressive growth characteristic and warrant increased caution.[11] However, these observations have not been validated in clinical

trials. A further possibility is that larger tumours have become more integrated with the body, with a wider zone of cytokine and humoral influence on the surrounding tissues. The significance of the relationship established between the tumour and the host tissues, and the role of the tumour microenvironment in influencing the potential for tumour recurrence after surgery, will be discussed in more detail in a subsequent section of the discussion.

Palpable characteristics: The results of the current study suggest that a difference in the mobility of the tumour may help predict the subsequent behaviour of the neoplasm. It was shown that STS that feel 'fixed' to underlying tissues on palpation had significantly increased rates of recurrence and reduced survival times compared to those tumours that were freely mobile. Other authors have not identified tumour fixation to be predictive of outcome,[4] so this finding needs to be validated in a prospective setting to see if it can be used to predict prognosis or guide resection margins. One reason why a STS that is more fixed to the underlying tissue could recur more commonly after surgery may reflect, again, the challenges an inexperienced surgeon can face in maintaining a consistent dissection boundary about the tumour, particularly if the surrounding tissues are more adherent to the tumour than normal. This increased surgical difficulty may lead to undocumented compromises in parts of the dissection whereby a surgeon may start out with a certain boundary about the mass but their surgical margins gradually shrink closer and closer to the tumour interface during the surgery as they try and find a comfortable dissection plane. This may lead to portions of microscopic tumour being inadvertently

left behind in some areas of the wound - potentially leading to recurrence - even though the surgeon will have documented that they removed the tumour with wide margins. Nevertheless, there are other explanations to explain why tumour mobility may impact on risks of tumour recurrence that are unrelated to surgical margins. For example, it is possible that STS that feel more fixed have a different tumour microenvironment that causes them to be more infiltrative or enables greater migration of tumour cells into the periphery. Similar effects have been described in human breast cancer, where diseased tissue can be 10-times stiffer than normal breast. This increase in stiffness is contributed to by increased levels of the enzyme lysyl oxidase (LOX) in the stroma. This increase in LOX causes the tumour-associated collagen to become linearised through increased cross-linking between the molecules. The cross-linked collagen bundles tend to be quite stiff and provide an effective highway for tumour cells to migrate along. This "highway" can facilitate their migration through the peri-tumoural tissues and ultimately into the vasculature and lymphatic circulation.[27]

Tumour grade, mitotic rate and necrosis: In the current study, the histological grade of the STS was found to be significantly associated with both local tumour recurrence and survival. Grade 2 and grade 3 STS were almost 2 and 6 times, respectively, more likely to cause the death of the dog compared to grade 1 tumours. Similar hazard ratios were also reported for the risk of local recurrence for both of these higher grade tumours. An association of histological grade with patient outcome has been reported by other authors, but more commonly due to a higher risk of metastatic disease. Rates of metastatic spread for grade 3 STS have been reported to be 22-

44%,[7, 28] compared to just 7-8% for grade 1 and 2 tumours.[29] It should be noted that the precision of these figures is poor due to the inadequacy of data collection on metastatic disease in all patients, a factor that will be examined in more detail later. An association between tumour grade and local recurrence of the tumour following surgery has been less well described. Only one study has demonstrated a correlation between the histologic grade of the tumour and the risk of recurrence for marginally excised subcutaneous STS.[30] In that study, only 7% (3 of 41) of low grade tumours recurred after marginal excision compared with 34% (14 of 41) and 75% (3 of 4) for intermediate and high grade tumours, respectively.

Because grade is an aggregated score of several elements of tumour biology, including differentiation, mitotic index (MI) and the presence of necrosis, it is not surprising that some of these individual measures were also significantly associated with disease outcome. In the current study, both a high MI (>20 mitoses per 10 high power fields) and a tumour that was more than 50% necrotic on histologic examination were associated with a significant increase in local recurrence and tumour-related death. The MI is a measure of proliferative activity within the tumour, and a high rate of cellular turnover is commonly associated with increased (and earlier) rates of tumour recurrence, higher rates of metastasis and reduced overall survival for many tumour types, including STS.[4] In one study on canine STS, MI was the only factor to be significantly prognostic for developing metastatic disease on multivariate analysis; in that study, dogs with an MI of >20 were 5 times more likely to develop metastasis than dogs with an MI of <20.[7] The role of MI in predicting local recurrence has only been reported in one previous

study, where almost two-thirds (63%) of tumours with an MI >9 recurred, while only a quarter of the STS with an MI of less than 9 recurred,.[10] Because MI is a measure of cellular turnover within the STS, it follows that a highly proliferative tumour will show more aggressive tendencies, and thus may respond to surgery more poorly. It is also possible that the increased drive for a sarcoma cell to proliferate reflects an increased concentration of growth factors and tumourigenic cytokines around the tumour, which creates a microenvironment favourable for tumour progression.

Although high levels of necrosis within the tumour has been associated with reduced survival and shorter disease-free intervals in several studies, this is the first veterinary study to show a significant relationship with increased local recurrence. An association between the presence of gross necrosis within the tumour and poorer patient outcome was first noted in human STS in 1984;[31] in that study the authors noted that patients with moderate and marked necrosis had a much poorer survival than patients with absent or minimal necrosis. In veterinary studies, one study has shown that tumours with >10% necrosis were 2.8 times more likely to lead to death of the dog than tumours with less than 10% necrosis.[7] It seems counter-intuitive that a tumour that appears to be dead on microscopic analysis is actually more aggressive than a tumour that does not have these necrotic areas. Reasons why this dichotomy could occur are explored in more detail later in this thesis, but it may relate to the effects of hypoxia and adaptive evolution by the tumour cell.[32] Hypoxia will develop within a growing tumour if it is unable to develop the necessary vascular supply to support its continued expansion. If a tumour proliferates too quickly, the cells will become

increasingly isolated from the existing vasculature, and may extend beyond the limits of oxygen diffusion. If the cancer cells are to survive in this hypoxic environment, they must either drive angiogenesis to increase the delivery of oxygen and nutrients to the growing tumour or adapt their metabolism to allow continued survival in the suboptimal conditions. Clonal evolution will probably favour cancer cells that are able to upregulate the necessary hypoxia response genes and pro-inflammatory genes that will support their survival in this challenging environment.[32]

While an increased proportion of tumour necrosis was found to be significantly associated with increased recurrence and reduced survival in the current study, it should be recognised that the evaluation of necrosis within a STS can be problematic. Necrosis is often not diffusely present within a neoplasm so examination of just a portion of a large neoplasm can either over- or underestimate the true proportion of necrotic cells within the entire tumour. This sampling error is often made worse because histology technicians are routinely trained not to trim necrotic areas of neoplasms in for histological evaluation.[33]

Histologic type: All canine STS have traditionally been considered as a single group for prognostic purposes.[4] However, evidence from human STS suggest that individual STS types will exhibit differences in local invasiveness, metastatic potential and recurrence.[34, 35] In canine STS, evaluation of differences in outcome between various histologic subtypes has been limited by small numbers of STS studied and a lack of consistency in the histological classification of STS by veterinary pathologists. In the current

study, 2 of 22 (9%) PWT developed local recurrence compared to 21-30% recurrence rates for PNST, myxoma and fibrosarcoma. This finding was not statistically significant, but this may be due to insufficient numbers of each tumour subtype. The possibility that PWTs may be less aggressive than other STS subtypes has been suggested by other authors.[4] It should be noted, however, that one of the characteristics hallmarks of a STS is the difficulty in consistently distinguishing the individual subtypes that make up this group using light microscopy alone. The histologic classification of STS subtypes is subjective with no clear published guidelines, leading to uncertainty and inconsistency between pathologists. The differences in behaviour between different STS subtypes in the current study could have been due to the pathologist interpreting histological features of aggressive behaviour more with some STS subtypes. For example, a neoplasm with greater MI or invasiveness may be more likely to be interpreted as a PNST whereas a STS that appears histologically benign may be more likely to be interpreted as a PWT. Furthermore, while one pathologist may have confidence in their own ability to recognise characteristics that can distinguish individual tumour subtypes, there is no certainty that a group of pathologists would all agree on the same diagnosis. In one study, partial or complete disagreement on different diagnostic criteria was reported between two pathologists in over 50% of cases.[36] Major disagreements in histopathologic diagnosis occurred in 19 (37%) cases. In these 19 cases, a second-opinion interpretation prompted a change in the recommended staging tests (10 cases), treatment plan (19 cases) or prognosis (10 cases). In 21% of cases, a third-opinion provided yet another interpretation of the tissue sections, in disagreement

with the first two. Nevertheless, if the variance in behaviour of different subtypes evident in the current study could be validated and confirmed, this would suggest the current single surgical rule for the treatment of all STS may not be optimal. It may be that different surgical strategies for different STS subtypes could be more appropriate.

Local tumour recurrence was the most common cause of tumour-related death in this study. This is similar to previous studies of canine STS.[1, 6, 7, 10, 11, 15] As canine STS only rarely spread to other sites in the body systemic illness due to the cancer is generally uncommon and the death of most dogs with STS is by euthanasia due to the effects of the mass on mobility or other impacts on quality of life. Euthanasia may be chosen by clients because the costs of surgical management of a STS may exceed the owner's financial limits, or a dog may have concurrent conditions that limit its ability to tolerate surgery. Therefore, if recurrence of the tumour occurs after surgery, the owners may choose not to proceed with any further treatment. In the current study, referring veterinarians were only asked to classify the cause of the death in a binary manner, i.e. was the STS a cause of death or not. In reality, a decision to perform euthanasia may be influenced by a combination of factors, including the presence of concurrent disease conditions. In a retrospective study such as this, it can be difficult to truly determine the precise cause of death, as the individual characteristics of the decision are not evident.

In the current study, it is worth noting that tumour recurrence did not always lead to death, with around a half of dogs with tumour recurrence dying due to

non-neoplastic causes. The reasons why some dogs had tumour recurrence but did not die of their tumour is difficult to determine because the necessary information was not requested in the questionnaire. For example, these dogs could have died due to other causes while the tumour was re-growing or the tumour may have been kept under control by repeated palliative excision, with this fact not being reported by the veterinarian in the questionnaire. Cavanaugh and others (2007) reported that repeated marginal (or intra-lesional) surgery can be effective in maintaining control of recurrent STS,[2] and this can be an effective palliative strategy in maintaining control in affected dogs. Although the results of the current study suggest that canine STS may not result in death even after tumour recurrence, further study is required to determine the precise reasons for this.

There are several limitations to retrospective analyses and long-term follow-up studies such as this. These limitations include: i) the accuracy and reliability of the data collected by questionnaire; ii) the impact of missing or absent data; and iii) the deficiencies of margin analysis by histology.

All of the clinical data used in the current study was obtained by questionnaire, with veterinarians asked to provide answers to questions many years after the original surgery. These questions included specific comment about the characteristics of a tumour, as well as the clinical investigations performed and the surgical strategy. In many cases, the questionnaire may have been completed by a veterinarian who did not perform the original surgery. It is likely that recollections about a patient would have required a reliance on clinical notes taken at the time of the

original procedure, which may not have contained all of the information in the detail requested in the questionnaire. There is also a potential for reporting bias, particularly with the description of surgical margin as some veterinarians may not want to admit that they had removed tumours with surgical margins that were less than those recommended by conventions of the time. It is therefore difficult to determine the veracity of the data obtained. This means that some of the observations relating to tumour characteristics (e.g. size, palpable findings) and the precise surgical margins employed may not be accurate.

For some sections of the questionnaire, veterinarians were unable provide a specific response, probably because this information was lacking in the clinical records. For that reason, information on tumour size and palpable characteristics was unknown for about a third of the cases in the current study. The extent of surgical margin used was also unknown in 16% of cases.

The inclusion criteria for the current study ensured that data on patient survival and tumour recurrence was more complete, with no unknown observations. For survival analysis, more than 80% of the dogs were known to have died during the study period, with a date of death provided from the clinical notes. For the remaining animals, it was presumed they were still alive, with the censor date for analysis determined to be the last date the dog was seen by their veterinarian. However, it is possible these dogs may have died at home with their death not reported to their veterinarian, or they had been presented to another veterinarian after this period with tumour recurrence. This lack of information could have impacted the final analyses.

As discussed above, the influence of local tumour recurrence as an actual cause of death is also open to question. Owners may make an end-of-life decision for their dog for a variety of reasons but may choose to blame the recurrence of the cancer even if it was not actually influencing the dog's lifestyle or quality of life directly at that time.

Because this was a retrospectively performed study, there was no standard protocol for the diagnostic examinations performed prior to surgery, and also no procedure for consistent follow-up of the patient after surgery. The impact of this limitation is mostly influential on the interpretation of metastatic disease, both at the time of the original surgery and also as a possible cause of death. In the current study, very few dogs had any form of imaging to assess the potential for metastatic disease prior to surgery being performed. It is likely the significance of this omission on the overall conclusions will be minimal, as it is recognised that the incidence of detectable metastasis at the time of original diagnosis and surgery of a STS is actually very low. In one study, metastatic lesions in the lung were found in less than 7% of grade 1 and grade 2 STS at the time of presentation for surgery.[37]

Metastases were identified in 28 dogs in the current study, with a further 12 dogs having both local recurrence and distant metastatic disease. Metastasis was considered to be the cause of death in 24 (60%) of these 40 dogs. The accuracy of this figure is questionable, as not every dog in the study was subjected to the same degree of post-operative examination. It is possible that the incidence of metastasis has been underestimated, because not all veterinarians evaluated dogs for metastatic disease following surgery or when

euthanasia was performed. Conversely, there is also a chance the incidence of metastasis has been overestimated. This is because a full post-mortem examination or histologic confirmation of the metastatic lesion was performed in less than 10% of cases where metastasis was suspected. In the 21 of 40 cases where apparent metastatic lesions were discovered on imaging studies, the veterinarians completing the questionnaire perhaps made an assumption that the prior STS was the primary source. In a small number of cases, a presumption of metastatic disease was made simply because the dog had developed acute neurological signs and spread of the STS to the brain was suspected. Of course, in an elderly patient, there are many potential sites of primary neoplasia that may be occult to physical examination, so the previous STS may not have been a contributing factor in the newly discovered metastatic disease at all. However, when such a patient presents to a veterinarian with an acute deterioration in their quality of life, and an end-of-life decision is being discussed with the owners, it can often be comforting to provide an explanation for the sudden demise of their loved pet. The possible spread of a cancer that was known to be malignant is well-understood by pet owners, enabling them to accept a decision for euthanasia.

Another important limitation of the current study was the inability to complete a histological review of surgical margins. This was often because the veterinarian had only submitted a portion of the mass, or the orientation of all sections of the tumour processed by the laboratory was not available. This lack of evidence may be critical as it is generally recognised that the discovery of neoplastic cells extending to tissue margins on histology is an important predictor for tumour recurrence.[1, 3, 4, 22, 38-41] In one study, dogs with

incomplete margins were more than ten times more likely to experience tumour recurrence than dogs where histological margins showed no evidence of residual tumour. However, the true impact is actually difficult to quantify. In a previous study,[3] incomplete margins were recorded in a third of cases, and this finding was associated with significantly shorter disease-free intervals (P=0.02). However, in that study, 19 of 34 (56%) of dogs with an incomplete histological margin did not develop a recurrence during a median follow-up of 2011 days (range 258-3486 days).[Chase D, unpublished data] Similar findings have also been reported for human STS. In one large series of 2084 patients, 72% of patients with positive margins exhibited no recurrence with a median follow up of 50 months.[42]

Although recognised as a limitation, the lack of standardisation of diagnostic and treatment protocols for patients in the current study actually provides a valuable insight into the strategies of first opinion veterinarians when dealing with a mass on a dog. It is concerning that more dogs had pre-operative blood tests performed for anaesthetic purposes than had any investigations directly related to understanding identity of the mass about to be operated upon. In only 20% of dogs was any attempt made to interrogate the mass using fine needle aspiration, and in many of these cases the veterinarians acknowledged this test was performed to rule out common tumours such as lipoma or mast cell tumour, rather than to identify a potential sarcoma. Less than 5% of dogs had a definite diagnosis of sarcoma before surgery. These findings are of interest and could be used to assist with the development of new treatment recommendations for STS following on from this study. Historically, there has been a tendency to encourage veterinarians to pursue

aggressive surgical margins for all soft tissue sarcomas in the belief that this would translate to improvements in survival and curative outcome. However, the results of the current study suggest that it is not the extent of resection that is the principle determinant of outcome, with aspects of STS biology (e.g. grade, % necrosis etc.) playing a more important role in influencing the potential for an individual tumour to recur. It is interesting to note that when a STS was suspected from pre-operative biopsy or cytology in the current study, there was a tendency for a wider surgical excision to be performed, including more dogs undergoing amputation procedures. However, outcomes for these patients were no different than the remainder of the population, with similar rates of local recurrence. The reason for this is not clear, but it does support the notion that some patients can be overtreated for their tumour.

One discovery that was evident from the current study was that many veterinarians choose to operate on a mass without any determination of whether or not the mass is neoplastic. Surgery was therefore performed without any consideration of whether the chosen surgical plan would be appropriate for the mass, or whether this plan could actually influence the success or failure of the surgery. In humans, such an 'unplanned' excision of a mass has been shown to detrimentally affect the long-term outcome for that patient if it is subsequently found to be a STS on histology. In one study, overall disease-free intervals and survival times were reduced for patients after an unplanned excision, even when that patient subsequently received appropriate curative intent surgery, radiation therapy and chemotherapy for their tumour.[39] Comparable veterinary studies are not available; one

author reported recurrence rates of 15% after re-excision of an inadequate resection of soft tissue sarcoma,[23] but no control population was available in this study so the actual benefits of this second intervention are unknown. However, reoperation is not always an option for every patient or their owner, which emphasises the importance of pre-surgical planning when dealing with a potentially malignant mass.

An explanation for why veterinarians may have an apparent blasé attitude to the importance of preoperative interrogation of a mass is that the surgical recommendations for many common malignant skin tumours such as STS and mast cell tumours are broadly similar.[43-45] Anecdotal observation suggests that many veterinary surgeons who do not have a special interest in cancer surgery will simply remove a mass along with an unmeasured boundary of skin and subcutaneous tissue. The extent of this tissue boundary will be influenced by their surgical confidence, and the anatomical limitations of the body part involved. This failure by veterinarians in first opinion practice to implement surgical guidelines that have been published in multiple textbooks is supported by the results of the current study where the majority of resection margins were described as marginal or local only. It is possible this complacency towards surgical planning is reinforced by the types of cases being managed in first opinion practice. Because the majority of STS are low-grade, the current study suggests that veterinarians working in first opinion practice actually achieve good long-term control in more than 80% of their patients. This low frequency of treatment failure may thus be insufficient to cause a veterinarian to reconsider their normal surgical strategy.

Importantly, although the results of the current study suggest that the extent of resection performed about a STS does not influence the disease-free interval or overall survival, this does not imply that surgical margins should be reduced in all cases or that wide margins are unnecessary. Because of the selection bias that is present it is unsurprising that surgical outcomes are better for first opinion veterinarians when they tend to operate on a higher proportion of low grade tumours. Current evidence suggests there is a spectrum of biologic behaviour of soft tissue sarcomas, with some responding favourably to narrow resection margins, whereas others will have a tendency to recur, almost in spite of the surgical margins employed. If this were true, this would suggest that an interrogation of the STS prior to surgical excision could be useful to help identify potential indicators of behaviour and therefore the most appropriate surgical margin to use.

Unfortunately, there is no current consensus on how to determine the best margin for each individual tumour. As discussed in the literature review, an identical debate on the surgical margins that are required to achieve adequate control of STS has occurred in the human literature, without a consensus being established.[46-49] One study analysed outcomes for 1261 patients with extremity STS over a 20 year period, and concluded that the prognosis for patients had not improved at all, indicating that current surgical strategies had reached the limits of efficacy.[50] For the human STS surgeon, the question of "how much margin is required" has largely been resolved by the incorporation of radiotherapy into most standard treatment protocols, as evidence demonstrated that the combination of surgery with adjuvant or neoadjuvant radiotherapy allowed surgical margins to be safely reduced

without compromising tumour control.[51] In veterinary oncology, the routine incorporation of radiotherapy is unlikely to become commonplace due to the combination of cost, limited access and other logistical reasons. Surgery will therefore remain the predominant weapon in the control of localised cancer. It will therefore be important to try and develop the ability to predict which STS have a higher tendency to recur after surgical excision, as this information would greatly help treatment planning.

In conclusion, the results of the current study pose an important question: why does outcome not always improve with an increasing resection margin? Some authors have questioned the limitations and reliability of margin interpretation in tumour histopathology and it is certainly possible these factors do play a significant role in confounding this issue [33, 52]; these topics were reviewed in the literature review. However, another important consideration is the structure of the pseudocapsule and the tumour microenvironment which will influence tumour biology and the potential for recurrence after surgery.[53, 54]

As discussed in the literature review, the pseudocapsule of a STS is a renowned feature of this tumour type.[49] While the pseudocapsule may appear to be a distinctive fibrous boundary between the tumour and the body, it is generally not considered to be an effective barrier to tumour cell migration into the surrounding tissues. However, in some tumours the fibrous pseudocapsule may actually provide an effective barrier against tumour growth and infiltration but this probably holds true for a proportion of (mostly) low grade lesions only.[49, 55, 56] In those instances, successful

local control could indeed be achieved with excision of the mass including a narrow rim of normal tissue; the results of the current study support this possibility. However, in higher grade tumours, there is an ill-defined area surrounding the pseudocapsule that contains diffusely spread clusters of neoplastic cells and a permissive stromal microenvironment. Tumour recurrence may be more likely to occur if the plane of surgical excision passes through this area. In light of these findings, it was decided that the next phase in this thesis would be investigate potential biomarkers that provide surrogate evidence of tumours that have a higher tendency to recur after surgery.

3.5 Conclusion

There were three broad aims for this study. Firstly, to determine the outcome for a large cohort of dogs diagnosed with STS that were managed exclusively in first opinion practice. The results of this study suggest that outcomes for dogs treated with STS in first opinion practice have improved since Bostock and Dye published the initial study on this tumour type over 30 years ago.[10] While this is likely to be primarily due to the inclusion of dogs from primary, rather than referral, veterinary practice it is also possible that factors such as earlier patient presentation, better patient selection and a bias in reporting patient populations may also have contributed to the better prognosis for canine STS observed in the current study.

The second aim was to evaluate the clinical and histological features of the STS to determine if any of these features was correlated with differences in

survival times. Results from the current study suggests that important factors in predicting prognosis included the age of the patient, tumour size, the palpable characteristics of the tumour, grade and the histologic type. These features were all found to have prognostic significance for either patient survival, tumour recurrence or both.

The third and final aim was to establish an archive of histological tissue from these patients which could be used for novel biomarker discovery as described in later chapters of this thesis. This was achieved and these cases were used in the following chapters to try and identify more accurate biomarkers to help prevent recurrence of a canine STS.

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Chapter 4:

Prognostic markers in soft tissue sarcoma: an immunohistochemical study

4.1 Introduction

The results of the previous chapter generated an important question: why does the outcome for dogs with soft tissue sarcoma (STS) not always improve with an increasing resection margin? It would seem reasonable that the more tissue removed around the tumour the less likely it should be for that tumour to recur. However, the previous study did not identify a statistical correlation between the extent of the resection margin and local recurrence ($p=0.8$), suggesting that factors other than the surgical margin may have an influence on the outcome.

While the evidence from the previous chapter suggests that some STS could be safely resected with smaller margins without increasing the risk of recurrence, the data also suggested that wider surgical margins may still be required to obtain adequate local control for other tumours. It is likely that the appropriate resection margin is variable between different STS, with certain aspects of tumour biology influencing the potential for recurrence after surgery.[1, 2] Given this variance, it would be helpful to identify features within a STS that could enable a surgeon to determine if an individual

tumour requires narrower or wider surgical margins to prevent recurrence after surgery.

For a microscopic cluster of cancer cells to progress in size to a clinically relevant neoplasm, it must have established a relationship with the host.[3, 4] One of the most important interactions that must occur between an emerging tumour and the body includes the development of a supporting network of blood vessels, a process known as angiogenesis.[5] Sustained angiogenesis is recognised as one of the hallmarks of cancer,[3] and is known to play an important role in tumour progression and the development of metastasis.[5] A variety of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), placental growth factor, transforming growth factor-β (TGF-β) and others have been shown to not only mediate the migration of microvascular endothelial cells, but to encourage their proliferation and formation into microvessels about the tumour.[6]

For the current study, two important angiogenic molecules – VEGF and decorin - were selected for investigation as possible prognostic markers in canine STS.

Vascular endothelial growth factor is considered to be a major driver of tumour angiogenesis [8]. Increased VEGF expression is reported to be a negative prognostic factor for a wide range of tumour types in humans, including breast cancer,[7] colorectal cancer,[8] ovarian carcinoma,[7] renal and bladder carcinoma,[9, 10] gastric carcinoma,[11, 12] osteosarcoma,[13] soft tissue sarcoma[14-17] and malignant effusions.[18, 19] In humans with

STS, higher serum concentrations of VEGF have also been correlated with an increased development of local recurrence and metastasis.[20] In the dog, VEGF has been studied in several canine tumours, including nasal tumours,[21] mast cell tumours,[22, 23] thyroid tumours,[24] haemangiosarcoma,[25, 26] central nervous system tumours,[27] mammary tumours,[28, 29] and soft tissue sarcoma.[30-33] For canine STS, positive VEGF immunostaining was identified in about 65% of tumours,[32] but a correlation with survival data has not been performed. In another canine study, the serum concentration of VEGF was shown to reduce following excision of the STS, suggesting the tumour was contributing directly to the increased VEGF production.[33]

Decorin is an important extracellular matrix protein belonging to the small leucine rich proteoglycan family.[34-36] Decorin interacts with several growth factors including members of the TGF-β family, FGF, tumour necrosis factor-alpha (TNF-α) and PDGF.[35] Decorin prevents angiogenesis in a variety of tumour cell lines,[37] and tissue levels have been shown to correlate inversely with the extent of vascularisation in human vascular tumours.[38] Decorin is primarily synthesised by fibroblasts located in the stroma, and production of decorin by a neoplastic cell appears to be extremely rare.[39] However, tumour cells have been shown to produce soluble factors that can actively suppress the production of decorin by stromal myofibroblasts.[40] Down-regulation of decorin expression in tumours has been demonstrated in several human cancers including breast, endometrial, ovarian and lung.[35, 36, 41-44] Epigenetic regulation of decorin gene expression has also been demonstrated in colon cancer.[42] For

humans with STS, lower decorin concentrations within the tumour are associated with a shorter disease-free ($p<0.05$) and overall-survival rates ($p<0.05$).[45] There have been no published studies on the influence of decorin and cancer in the dog.

The aim of the current study was to use immunohistochemistry to identify whether these two stromal proteins – VEGF and decorin - are present within canine STS that have been previously resected. Analysis was then performed to determine if immunostaining characteristics were associated with different rates of tumour recurrence and/or patient death. It was hypothesised that increased immunostaining for VEGF within the tumour would reduce patient survival times and increase the rate of tumour recurrence following surgical resection. Conversely, high levels of decorin within the tumour was hypothesised to improve survival times and reduce recurrence rates of STS after surgery.

4.2 Materials and Method

4.2.1 Patient selection

This immunohistochemical study was performed using 100 cases selected from the tissue archive of 350 formalin-fixed paraffin-embedded (FFPE) specimens that had been established in the previous chapter. Cases were excluded from selection if the grade of the tumour or status of local recurrence was unknown. Because the archived population was known to be heavily biased towards grade 1 tumours, stratified random sampling was used to ensure that the cohort of 100 patients selected for immunohistochemical

study would contain a proportion of grades roughly equivalent to the cases where local tumour recurrence developed within the parent population. This was achieved by firstly determining the proportion of local recurrence that occurred for grade 1, grade 2 and grade 3 STS within the parent population, after the excluded cases were removed. This calculation was then used to decide the number of cases from each grade that should be selected from the parent population to create a total cohort of 100 dogs. The random function within R (R v 3.2.3, R Development Core Team) was used to select patients from each grade, based on these previously calculated proportions.

Clinical details about each STS had been determined by questionnaire, as detailed in the previous chapter. Follow-up information available for each tumour included the size, location and palpable characteristics (fixed or mobile), as well as the current status of the dog including the period in days until the development of local recurrence, metastasis, or death. Surgical resection margins were defined as marginal (neoplastic cells were visible adjacent to the margins), local (less than 3cm), wide (3cm or more) or amputation. If this information was not available in the clinical records, the resection margin was defined as unknown. The histological diagnosis and grading characteristics, including information on the degree of differentiation, percentage necrosis and mitotic rate of each STS, had been previously reviewed by a single pathologist according to published guidelines.[32]

4.2.2 Immunohistochemistry

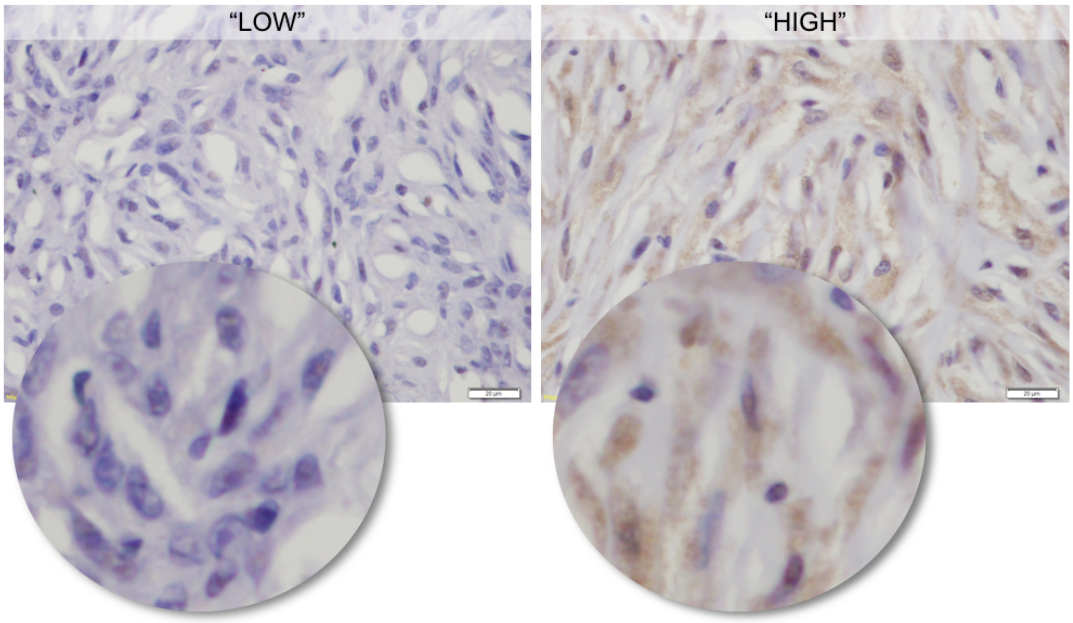
Tissue sections (5µm) were obtained from each tumour and mounted onto positively charged glass slides. Sections were dewaxed in xylene and rehydrated in a graded alcohol series and equilibrated in phosphate buffered saline. Antigen retrieval was performed in a decloaker (Biocare Medical, Pacheco, CA) at 100°C for either 20 mins (VEGF) or 2 mins (decorin) in a buffer solution (EnVision™ FLEX Target Retrieval Solution (high pH), Dako Australia Pty. Ltd). Immunohistochemistry was then performed using a Sequenza Immunostaining Center (Thermo Fisher Scientific, UK). Endogenous peroxidase activity was blocked using Peroxidase-Blocking Reagent (EnVision™ FLEX, Dako Australia Pty. Ltd) for 15 mins. Tissue sections were incubated overnight with a 1:300 dilution of mouse antihuman VEGF polyclonal antibody [0.33µg /ml] (VEGF (A-20) sc-152: Santa Cruz Biotechnology Inc, Dallas, TX) or a 1:400 dilution of mouse antihuman decorin polyclonal antibody [0.25µg/ml] (Anti-DCN, Sigma-Aldrich Co, St Louis, MI). The specificity of these antibodies for the canine proteins has been previously reported so validation of these antibodies was not required.[46-49] Antibody detection was performed using diaminobenzidine (DAB) (Dako Australia Pty). Positive and negative controls were used for each batch of slides. Positive control tissues for VEGF were FFPE sections of canine haemangiosarcoma; for decorin, sections of skeletal muscle were used. For negative control tissues, the primary antibody was omitted.

4.2.3 Evaluation of immunostaining

Each slide was assessed by light microscopy and immunostaining of either VEGF or decorin was determined. Immunostaining was only evaluated in areas of well-preserved tissue morphology and away from areas of necrosis, tissue edges and other artifacts. Two investigators reviewed all slides independently and were blinded to other features of the tumour. Where disagreement was present, consensus was achieved by joint review.

Immunostaining using anti-VEGF antibodies was scored using a modification of a previously reported method (Figure 4.1) [14] Briefly, tumours were scored based on the proportion of cells showing evidence of VEGF

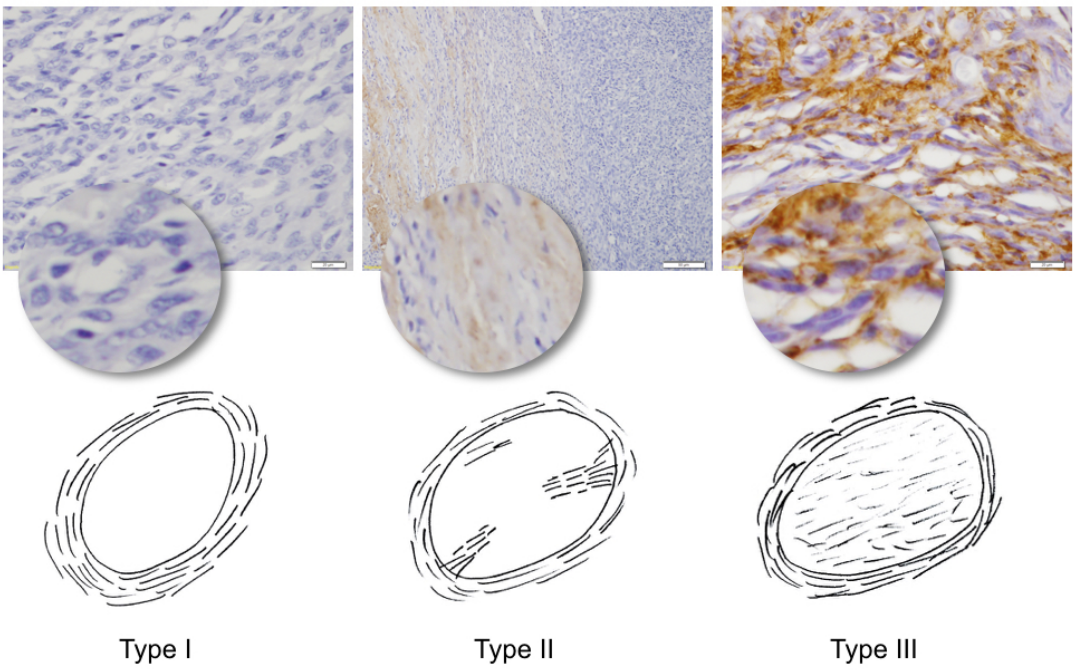
Figure 4.1:
Grading scale of immunostaining for vascular endothelial growth factor (VEGF). A low VEGF score was assigned if less than 75% of cells were immunostained. For a high VEGF tumour, more than 75% of the cells showed positive immunostaining.



immunostaining across 5 non-adjacent and non-overlapping fields. A tumour was classified as having "low VEGF" if less than 75% of cells were immunostained, whereas a tumour in which more than 75% of cells showed immunostaining was classified as having "high VEGF". Where distribution was not homogenous across the tumour fields, the highest score observed was assigned.

The presence of decorin was determined by evaluating the distribution of immunostaining within the tumour (Figure 4.2). A "type 1" pattern was assigned when decorin immunostaining was confined to the peritumoural

Figure 4.2:
Grading scale for decorin immunostaining. A type 1 pattern was assigned when decorin immunostaining was confined to the peri-tumoural margins only and no staining was visible within the tumour itself. A type 2 pattern was applied to cases where isolated islands of immunostained stromal tissue penetrated the tumour at various locations. For a type 3 pattern, decorin-labelled stroma saturated the entire tumour and intertwined closely about individual cells.



margins. A “type 3” pattern indicated that decorin-labelled stroma saturated the entire tumour and intertwined closely about individual cells while a “type 2” pattern was applied to cases where isolated islands of immunostained stromal tissue penetrated the tumour at various locations. When a STS showed little to no immunostaining within the tumour, the presence of intense immunostaining in the peri-tumoural tissues provided a good positive internal control.

4.2.4 Statistical evaluation

All statistical analyses were performed using SPSS Statistics (SPSS, version 26, IBM corporation). Local recurrence and death due to the effects of the tumour were defined endpoints of the study. Survival time was defined as a dog dying or being euthanased due to either local recurrence or metastasis. The disease-free interval was defined as the number of days from surgery until local recurrence was identified by the veterinarian. Any cases with an unknown finding within the category being analysed were not included in the statistical evaluation of that characteristic.

Chi-square analysis using Fisher’s Exact test was performed to evaluate variations in immunostaining according to age, sex, tumour size, location, palpable characteristics, surgical excision margins, tumour grade, presence of necrosis and mitotic index.

The Kaplan-Meier method was used to compare survival times to assess the significance of association between the immunostaining characteristics of a tumour with VEGF and decorin with the outcome measures of local

recurrence and tumour-related death. Univariate Cox proportional hazard analysis was used to assess the association between immunostaining characteristics and other clinically relevant variables described above against both survival time and disease-free interval. Hazard ratios (HR), 95% confidence intervals (CI) and their corresponding p-values were calculated. A value of $p<0.05$ was considered significant.

Immunostaining using both antibodies were also used to classify the tumours into six groups by combining prognostic scores from the most unfavourable to the most favourable, as follows: VEGF-high & decorin type 1; VEGF-high & decorin type 2; VEGF-high & decorin type 3; VEGF-low & decorin type 1; VEGF-low & decorin type 2; and VEGF-low & decorin type 3. Statistical analyses were repeated as above to determine differences between these six groups of immunostaining characteristics.

4.3 Results

4.3.1 Patient selection and demographics

From the original patient archive of 350 patients, 17 were excluded as local recurrence was unknown. A further 2 patients were excluded as tumour grade was undetermined. This left a population of 331 dogs. Within this remaining population, local recurrence occurred in 72 (22%) patients, comprising 42 (58%) in grade 1, 24 (33%) in grade 2 and 6 (8%) in grade 3. Using these proportions, 22 (22%) patients were selected from the parent population where local recurrence was recorded, comprising 12 (55%) grade 1, 7 (32%) grade 2 and 3 (13%) grade 3 STS. A further 78 (78%) patients were

then selected from the parent population where local recurrence did not occur, comprising 45 (58%) grade 1, 26 (33%) grade 2 and 7 (9%) grade 3 STS, creating a total cohort of 100 patients.

4.3.2 Determination of Immunostaining characteristics

Immunostaining for VEGF was interpretable in 82 dogs with STS. In the remaining 18 cases, artefactual defects or the lack of positive internal controls prevented interpretation. Details of the tumours included in this group are outlined in Table 4.1. Within this cohort, tumour size (p=0.04), palpable characteristics of the tumour p=0.004) and the development of local recurrence (p<0.0001) all had a significant influence on survival on Kaplan-Meier analysis. However, tumour grade and the histological diagnosis of the tumour were not influential on survival outcome. Only the palpable characteristics of the tumour were influential on the disease-free interval (p= 0.03).

The resection margins obtained about the tumour were not found to significantly influence either survival (p= 0.2) or local tumour recurrence (χ^2 = 7.0, p= 0.07) Because amputation could bias the potential for local recurrence, cases managed by amputation were removed from this analysis. In this remaining cohort, local tumour recurrence occurred in 12 of 26 (46%) of cases managed by marginal excision, 10 of 41 (24%) of cases managed with local excision, and 1 of 4 (25%) of cases managed by wide excision. However, resection margins were still not significantly associated with local recurrence (χ^2 = 3.5, p= 0.17). Of the 82 tumours where immunostaining could be

Table 4.1:

Results from chi-square analysis for different tumour characteristics and VEGF immunostaining

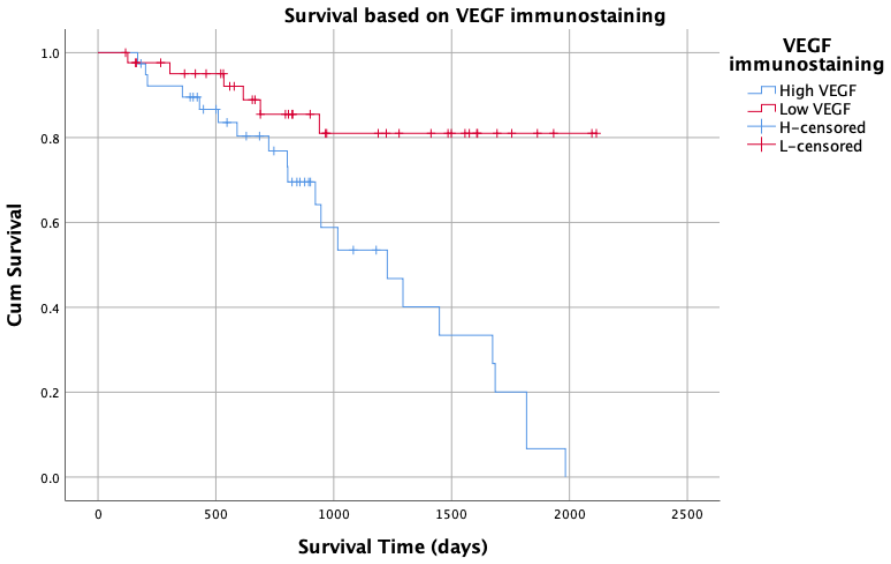
	VEGF (n=82)			
		Low	High	
	n (%)	N = 43	N=39	P value
Sex				0.65
Female	49 (60%)	27	22	
Male	33 (40%)	16	17	
Neutered				0.13
No	45 (55%)	20	25	
Yes	37 (45%)	23	14	
Tumour location				0.75
Head	4 (5%)	2	2	
Trunk	27 (33%)	16	11	
Limb	51 (62%)	25	26	
Size				0.31
<1cm	2 (2%)	1	1	
1-5cm	45 (55%)	26	19	
>5cm	21 (26%)	8	13	
unknown	14 (17%)	8	6	
Palpable				0.15
Discrete	32 (39%)	21	11	
Firmly adherent	45 (55%)	20	25	
Unknown	5 (6%)	2	3	
Grade				0.53
1	46 (56%)	25	21	
2	27 (33%)	12	15	
3	9 (11%)	6	3	
Degree of resection				**0.0003**
Marginal	26 (32%)	5	21	
Local	41 (50%)	30	11	
Wide	4 (5%)	3	1	
Amputation	7 (8%)	4	3	
Unknown	4 (5%)	1	3	
Diagnosis				0.11
Fibrosarcoma	20 (24%)	7	13	
Myxoma	1 (1%)	0	1	
Peripheral nerve sheath tumour	52 (63%)	29	23	
Perivascular wall tumour	9 (11%)	7	2	
Tumour cause of death				**0.0001**
No	55 (67%)	37	18	
Yes	27 (33%)	6	21	
Local recurrence				**0.0001**
No	56 (68%)	39	17	
Yes	26 (32%)	4	22	
Survival Time	days	days	days	
Minimum	117	117	168	
Maximum	2114	2114	1983	
Mean	907	961	847	

interpreted, 43 (52%) tumours were graded as low VEGF and 39 (48%) were high VEGF. There was a statistically significant association between VEGF immunostaining and resection margins, with 21 of 26 STS that had a marginal excision being classified as high VEGF ($p<0.0001$). All 5 of the STS that had more than 50% necrosis (score 3) had high immunostaining for VEGF. This compares with 12 of 23 (52%) and 22 of 54 (42%) STSs that had up to 50% necrosis (score 2) and no necrosis (score 1) respectively.

There was no association between VEGF immunostaining and the following characteristics: the sex ($p=0.7$) or neuter status of the dog ($p=0.1$), tumour location ($p=0.7$), tumour size ($p=0.3$), palpable characteristics ($p=0.1$), tumour histologic type ($p=0.4$), grade ($p=0.5$) or mitotic index ($p=0.2$).

Having a low VEGF immunostaining pattern was significantly associated with a longer overall survival time ($\chi^2 = 13.0$, $p = 0.0003$) (Figure 4.3). The

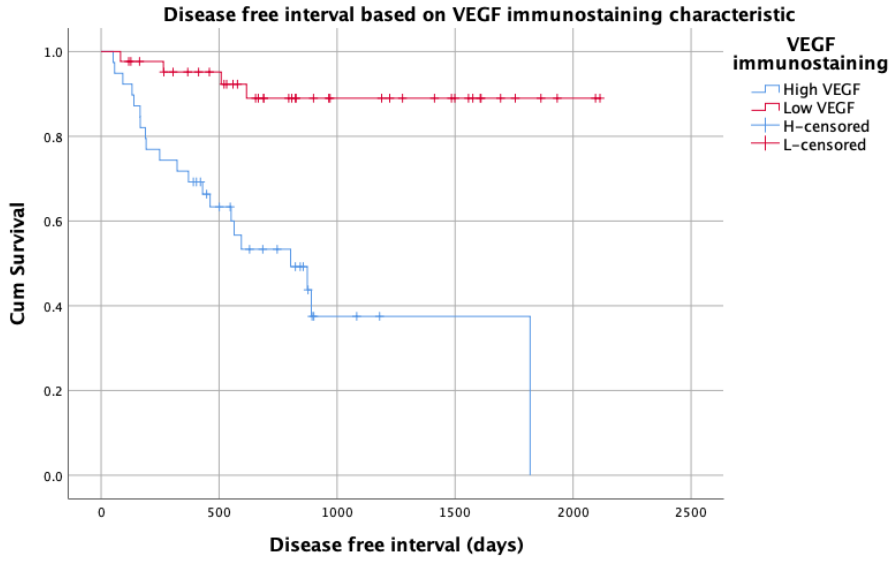
Figure 4.3:
Kaplan Meier graph of survival time for 82 patients with soft tissue sarcoma based on VEGF immunostaining (low and high).



median survival time for patients with a low VEGF could not be calculated as more than 50% of the dogs remained alive at the close of the study. Overall, 85% of patients with low VEGF remained alive more than 2 years after surgery, with 80% surviving 5 years or more. This contrasts with patients with high VEGF, where the proportion surviving at 2, 3 and 5-year intervals was 75%, 50% and 7% respectively. The median survival time for patients with a high VEGF was 1294 days (95% CI 774 – 1813 days). Having a high VEGF tumour increased the risk of death from the STS by a factor of more than four (HR 4.6 (95% CI 1.8-11.5, $p = 0.001$). On multivariate analysis, only high VEGF (HR 8.6, $p<0.0001$, 95%CI =2.8-26.4) was found to be associated with survival.

A STS with high immunostaining for VEGF was also significantly more likely to recur after surgery; (56% vs. 9%, $p <0.001$) (Figure 4.4). High VEGF immunostaining tumours were 7.3 times (95% CI 2.5 – 21.4, $p < 0.001$) more likely to recur than tumours with low VEGF. More than 90% of patients with a low VEGF STS remained disease-free at 2-, 3- and 5-yrs, compared to 51%,25% and 3% respectively of dogs with high VEGF STS.

Figure 4.4:
Kaplan Meier graph of disease-free interval for 82 patients with soft tissue sarcoma based on VEGF immunostaining (low and high).



4.3.3 Decorin

Decorin immunostaining could be evaluated in 83 cases. In the remaining 17 cases, artefactual defects or the lack of positive internal controls prevented interpretation. Demographic details of the tumours included in this group are outlined in Table 4.2. From Kaplan-Meier analysis, tumour size (p=0.05), palpable characteristics of the tumour (p=0.003) and the development of local recurrence (p<0.0001) all had a significant influence on survival within this cohort. However, tumour grade and the histological diagnosis of the tumour were not influential on survival outcome. There was no association between resection margins and survival or local tumour recurrence.

Of the 83 STS, 27 (32%) had a type I pattern, 24 (29%) a type 2 pattern, and 32 (39%) a type 3 pattern. Twenty-five of the 50 STS that were less than 5cm

Table 4.2:

Results from chi-square analysis for different tumour characteristics and decorin immunostaining

		Decorin (n=83)			
		Type 1 (n=27)	Type 2 (n=24)	Type 3 (n=32)	
	n (%)	n	n	n	P value
Sex					0.07
Female	47 (57%)	21	13	13	
Male	36 (43%)	6	11	19	
Neutered					0.9
No	40 (48%)	11	12	17	
Yes	43 (52%)	16	12	15	
Tumour location					**0.02**
Head	5 (6%)	1	1	3	
Trunk	28 (33%)	16	3	9	
Limb	51 (61%)	11	20	20	
Size					**0.03**
<1cm	0	0	0	0	
1-5cm	50 (60%)	13	12	25	
>5cm	21 (25%)	8	10	3	
unknown	12 (16%)	6	2	4	
Palpable					0.6
Discrete	31 (37%)	6	8	17	
Firmly adherent	45 (54%)	18	13	14	
Unknown	7 (8%)	3	3	1	
Grade					**<0.0001**
1	46 (55%)	6	11	28	
2	28 (34%)	13	12	3	
3	9 (11%)	8	1	1	
Degree of resection					0.3
Local	68 (82%)	21	20	27	
Wide	8 (10%)	3	4	1	
Amputation	7 (8%)	3	0	4	
Diagnosis					0.06
Fibrosarcoma	22 (26%)	11	2	9	
Myxoma	1 (1%)	0	0	1	
Peripheral nerve sheath tumour	52 (63%)	16	19	17	
Perivascular wall tumour	8 (10%)	0	3	5	
Tumour cause of death					**0.02**
No	61 (73%	15	18	28	
Yes	22 (27%)	12	6	4	
Local recurrence					0.55
No	62 (76%)	19	17	26	
Yes	21 (24%)	8	7	6	
Survival Time		days	days	days	
Minimum	117	126	163	117	
Maximum	1983	1993	1983	1900	
Mean	884	928	877	882	

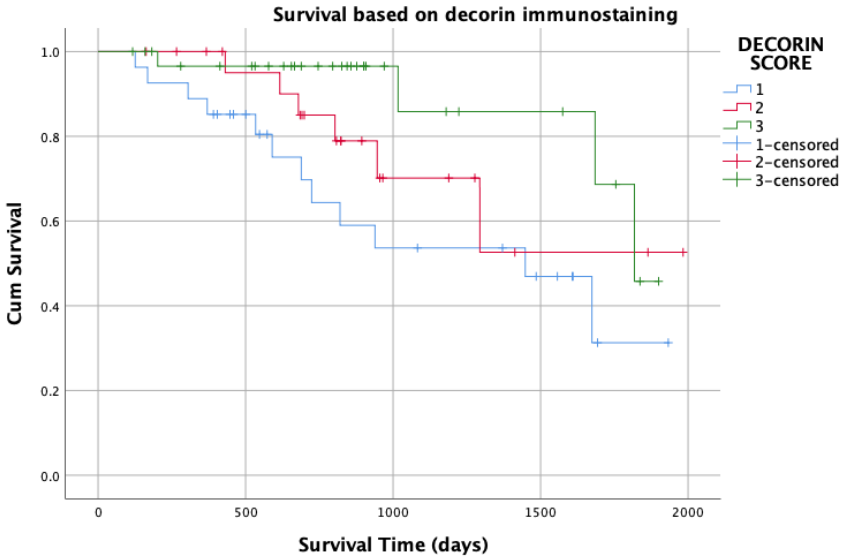
in diameter had a type 3 decorin pattern and this pattern was significantly more frequent in these smaller tumours than in STS that were greater than 5cm in diameter (3 of 21 (14%); p=0.02). Additionally, there was a significant association between the decorin immunostaining pattern and the extent of necrosis within the STS. Twenty-seven of 45 (60%) tumours that had low levels of necrosis displayed a type 3 pattern whereas in tumours that had >50% necrosis, all 5 displayed a type 1 pattern suggesting negligible decorin presence within the tumour.

The distribution of decorin immunostaining patterns was significantly different depending on the location of the STS (p = 0.03). For tumours of the trunk, 16 of 28 (57%) had a type 1 immunostaining pattern, compared to 1 of 5 (20%) and 11 of 51 (22%) of tumours of the head and limb respectively.

None of the PWT had a type 1 immunostaining pattern, compared to 10 of 20 (50%) fibrosarcoma and 17 of 53 (32%) PNST. This difference was statistically significant (p = 0.05).

There were no significant associations between the decorin immunostaining pattern and the sex of the dog (p=0.07), the neuter status of the dog (p=0.9), the palpable characteristics of the STS (p=0.6), the resection margins (p=0.3) or the mitotic index within the STS (p=0.1).

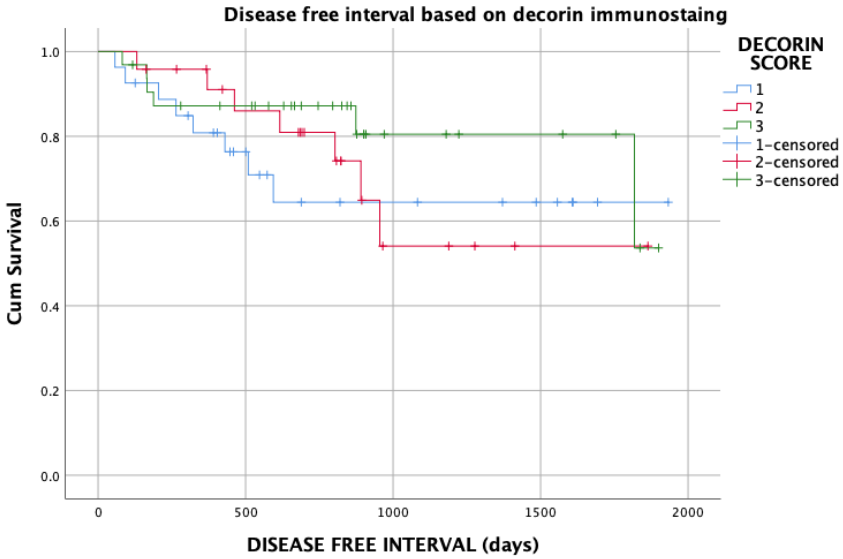
Figure 4.5:
Kaplan Meier graph of survival time for 83 patients with soft tissue sarcoma based on decorin immunostaining pattern (types 1 and 2 and 3)



For STS with a type 1 pattern of decorin immunostaining, 12 of 27 (44%) dogs died due to their STS, compared to 6 of 24 (25%) and 4 of 32 (12.5%) with type 2 or 3 patterns, respectively, a finding that was statistically significant. ($\chi^2 = 7.7$, p=0.02) (Figure 4.5).

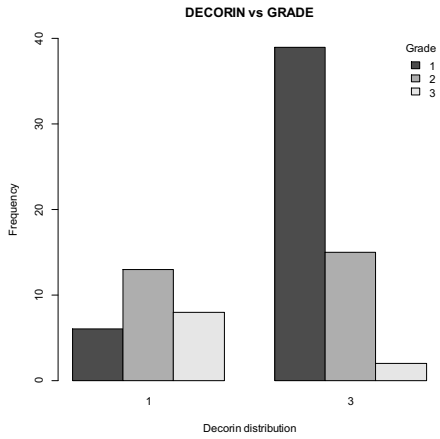
Local recurrence of the STS occurred in 8 of 21 (38%) with a type 1 pattern of decorin immunostaining, 7 of 21 (33%) and 6 of 21 (29%) with type 2 or 3 patterns, respectively. This was not statistically significant (p=0.5) (Figure 4.6).

Figure 4.6:
Kaplan Meier graph of disease-free interval for 83 patients with soft tissue sarcoma based on decorin immunostaining pattern (types 1, 2 and 3).



The decorin immunostaining pattern was significantly correlated with the histological grade of the STS with low-grade tumours more likely to have a type 3 pattern and high-grade tumours more frequently having a type 1 pattern (p <0.001) (Figures 4.7).

Figure 4.7:
Distribution of decorin immunostaining pattern according to tumour grade. Low grade tumours were more likely to have a type 3 decorin pattern.



4.3.4 Combined VEGF and Decorin

There were 71 cases for which both VEGF and decorin immunostaining could be interpreted (Table 4.3). Decorin and VEGF immunostaining were not correlated (p=0.9) (Figure 4.8). When the favourable and unfavourable extremes of the combined scores were compared, a STS with both a high VEGF and type 1 decorin distribution had a significantly lower MST than a dog with a STS with a favourable prognostic combination (i.e. low VEGF and type 3 decorin distribution) (1031 days vs. 1924 days, p <0.001). No tumour-related deaths occurred in 18 dogs with a low VEGF / type 3 decorin combination, compared to 6/12 (50%) deaths in STS with a high VEGF / type 1 decorin combination (log rank 16.7, p = 0.005). Similarly, only 1/17 (6%) STS with a low VEGF / type 3 decorin combination recurred, compared to 6/12 (50%) with a high VEGF / type 1 combination (log rank 22.3, p <0.001; Figure 4.9).

Figure 4.8:
Distribution of decorin immunostaining pattern according to VEGF score. No correlation was identified.

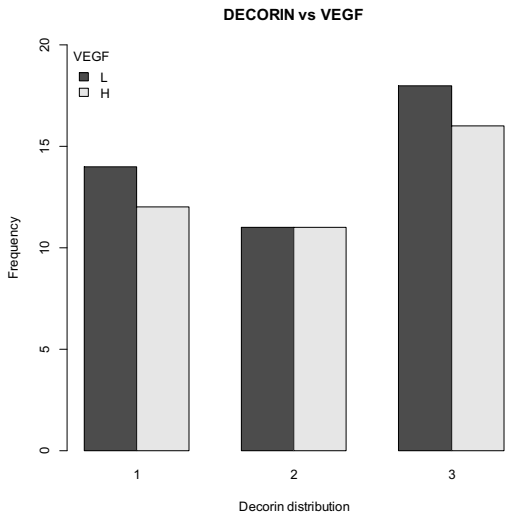
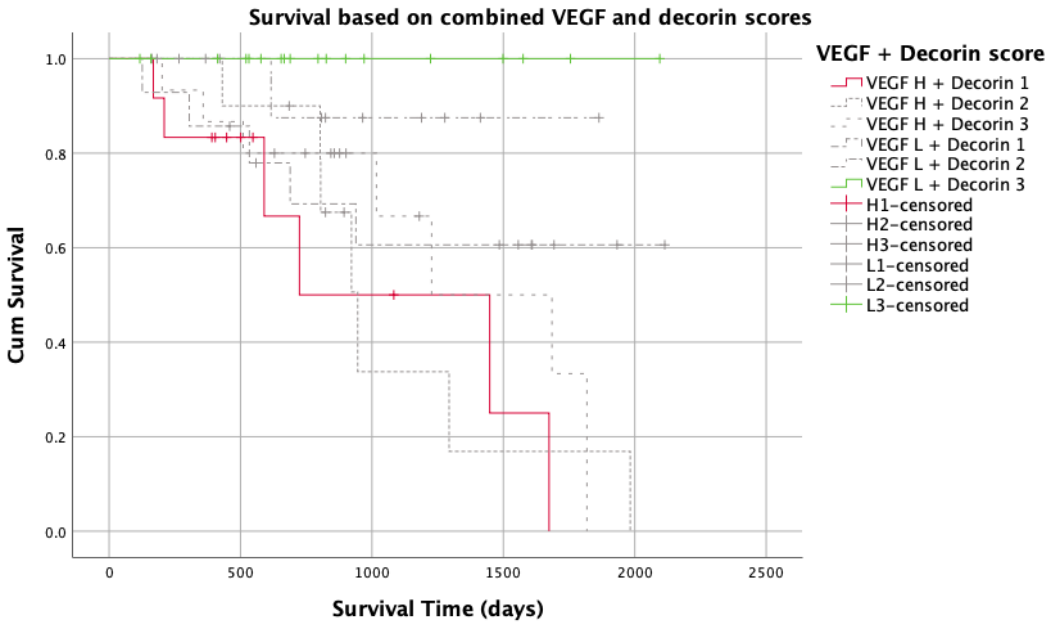


Table 4.3:

Univariate cox-regression analysis for survival and local recurrence for patients based on VEGF, decorin and combined immunostaining groups.

			Survival				Local tumour recurrence			
Immunostain n (%)			Disease free n (%)	Died from tumour n (%)	p-value	HR (95% CI)	No recurrence n (%)	Local recurrence n (%)	p-value	HR (95% CI)
VEGF (n=82)			55 (67%)	27 (33%)	>0.001	4.6 (1.9 - 11.5)			>0.001	7.3 (2.5-21.4)
	Low	43 (52%)	37 (45%)	6 (7%)			39 (48%)	4 (5%)		
	High	39 (48%)	18 (22%)	21 (26%)			17 (21%)	22 (27%)		
DECORIN (n=83)			61 (73%)	22 (27%)	0.041	0.5 (0.2 - 1.5) -	62 (75%)	21 (25%)	0.214	
	1	27 (32%)	15 (18%)	12 (14%)			19 (23%)	8 (10%)		
	2	24 (29%)	18 (22%)	6 (7%)			17 (20%)	7 (8%)		
	3	32 (39%)	28 (34%)	4 (5%)			26 (31%)	6 (7%)		
VEGF + Decorin					0.005		16 (100%)	0 (0%)	0.0005	
(Best)	L3	16	16 (100%)	0 (0%)			8 (89%)	1 (11%)		
	L2	9	8 (89%)	1 (11%)			9 (82%)	2 (18%)		
	L1	11	8 (73%)	4 (36%)			7 (50%)	7 (50%)		
	H3	14	7 (50%)	7 (50%)			4 (36%)	7 (64%)		
	H2	11	4 (36%)	7 (64%)			5 (50%)	5 (50%)		
(Worst)	H1	10	5 (50%)	5 (50%)						

Figure 4.9:
Kaplan Meier graph of survival time for 73 patients with soft tissue sarcoma based on 6 groups of combined VEGF and decorin immunostaining levels. The extreme prognostic groups (H1 and L3) are highlighted; survival times between these two groups are significantly different (1031 days vs. 1924 days, p <0.001)



4.4 Discussion

In the current study, a significant association was identified between high levels of VEGF within the tumour and higher rates of local recurrence of the STS after surgery. Additionally, high VEGF immunostaining within the STS was also associated with a four times higher risk of death from the tumour. This is the first study to demonstrate an association between VEGF levels and prognosis in canine STS. Only one other study investigating VEGF and canine STS has been performed.[32] In that study, VEGF immunostaining was observed in about 65% of STS, but the presence of VEGF was not investigated as a possible prognostic marker. In human STS, two studies have reported a positive correlation between increased VEGF expression and higher tumour

grade, but were unable to confirm an association with clinical outcome due to insufficient data.[50, 51] However, increased VEGF expression has been shown to be a negative prognostic factor for a range of other tumour types in both dogs and humans.[12, 18, 21, 23, 48, 50, 52-57]

The current study also revealed that reduced decorin within a canine STS was significantly associated with an increased chance that the dog will subsequently die due to the STS. While no statistical association was found between decorin pattern and local recurrence, this may have been due to the small patient numbers in this study. Decorin has not previously been investigated in canine tumours. There are also limited prognostic studies to evaluate the role of decorin in humans with STS, although the ability for decorin to influence the behavior of human cancer has been reported in several *in vitro* and *in vivo* studies.[37, 39, 43, 44, 58] In one study on human STS, decorin was assessed in 85 different tumours by real-time quantitative PCR and immunohistochemistry.[45] In that study, decorin expression was shown to vary according to histologic type; benign tumours such as lipoma and neurofibroma expressed higher quantities of decorin than more malignant types (liposarcoma and peripheral nerve sheath tumour). Low levels of decorin within the tumour were also associated with reduced disease-free ($p<0.05$) and overall-survival rates ($p<0.05$). In addition, decorin expression in recurrent or metastatic STS was lower than in the primary lesions, supporting a hypothesis that these secondary tumours have a more aggressive phenotype than the original primary tumour.[45]

In the current study, the prognostic potential of combining the immunostaining results of both VEGF and decorin was also evaluated. When VEGF and decorin immunostaining classifications were combined, the ability to identify subsets of tumours with very favourable or very unfavourable outcomes was improved. Thus, a STS with a combination of poor prognostic scores (i.e. high VEGF and type 1 decorin distribution) was more likely to recur or cause death of the dog compared to a STS with the most favourable combination of prognostic scores (i.e. low VEGF and type 3 decorin distribution). This finding supports a strategy where a suite of different prognostic markers could be used to better predict individual tumour behavior than relying on one single attribute alone.

The findings of the current study suggest that both VEGF and decorin can profoundly influence the behaviour of a STS. Both VEGF and decorin are extracellular matrix [ECM] proteins and these, and other ECM proteins, have been shown to be under- or over-produced in a number of different cancer types,[59-61] with varying influence on the progression of the tumour. To the author's knowledge, this is the first time that the presence of these proteins has been evaluated for their role as potential biomarkers in canine STS.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and has been described as an essential growth factor for vascular endothelial cells.[62] VEGF, along with a variety of other angiogenic factors such FGF, PDFG, placental growth factor, TGF-β and others, mediates the migration of microvascular endothelial cells, but also encourages their proliferation and formation into microvessels.[6] While the growth of a new vascular system is

fundamental for embryonal development and growth,[62] formation of new blood vessels is unusual in the normal adult animal. In an adult, angiogenesis will occur in the female reproductive system during ovulation, menstruation, and the formation of the placenta, but otherwise will only occur during wound healing or organ regeneration. However, angiogenesis is a vital process in the evolution of a tumour: if a neoplastic growth is to proceed beyond a 2mm cellular mass, it must develop its own vascular supply. This requires cooperation from the body's own resources.[5] As early as 1971, it was proposed that a tumour could be maintained in a dormant state simply by inhibiting angiogenesis.[5] However, it took another 25 years before the mechanisms that enabled this 'angiogenic switch' to occur were described,[63] with identification of the molecules involved in this process reported in 1997.[64]

In the current study, a significant association between the extent of necrosis within the STS and both increased VEGF and reduced decorin immunostaining was found. Soft tissue sarcoma with high levels of necrosis were found to have a less decorin immunostaining while tumours with low levels of necrosis were more likely to show type 3 decorin immunostaining. Smaller tumours were also more likely to have type 3 decorin immunostaining than larger tumours. Because necrosis is an indicator of a tumour that is poorly viable and larger tumours are more likely to have grown beyond the capacity of their innate blood supply, it seems reasonable to presume that the impact of hypoxia and its influence on angiogenic mechanisms is a probable explanation for these changes. The two ECM proteins examined in the current study - VEGF and decorin - were chosen

due to their known influence on angiogenesis.[36, 37, 62] Angiogenesis is controlled by a complex balance between stimulatory and inhibitory signals for blood vessel growth.[65] The influence that these two proteins have on angiogenesis are described in more detail below.

Stimulatory: Vascular endothelial growth factor is implicated as one of the major stimulatory factors in tumour angiogenesis.[62, 65] Increased expression of VEGF by tumour cells is influenced principally by hypoxia, but a number of other growth factors (e.g. TNF-α, TGF-β, epidermal growth factor (EGF) and PDGF, COX-enzymes) and oncoproteins (including ras, HER2, EGF and bcr-abl) are also involved in inducing VEGF expression. A tumour may also develop autonomous secretion of VEGF by epigenetic or DNA mutational change.

Inhibitory: Decorin is considered to inhibit angiogenesis by suppressing the production of endogenous VEGF by the tumour cell. Decorin is known to bind strongly with several growth factors within the ECM, including the TGF-β family, FGF, TNF-α and PDGF, amongst others.[35] Normal tissue levels of decorin allow the ECM to act like a sponge for these vital signaling molecules, creating a concentration gradient and providing some regulation to their availability for cell-signalling.[66] It follows that less decorin in the tissues will lead to higher concentrations of VEGF, TGF-β, FGF and PDGF being available in the extracellular environment, allowing for increased interactions with cellular receptors.[45] One study evaluated the difference in decorin expression between benign (i.e. haemangioma) and malignant vascular tumours (i.e. Kaposi's sarcoma and angiosarcoma).[38] In malignant

tumours, no decorin mRNA expression or immunoreactivity was detected while it was abundantly present in the benign tumours, particularly in the connective tissue stroma surrounding the clusters of intratumoural blood vessels. There were also fewer blood vessels present in the benign tumours. These results support the conclusion that decorin possesses a suppressive effect on tumour angiogenesis.

A reason why varying levels of these ECM proteins may influence the potential for local tumour recurrence may be explained by their potential influence on the tumour microenvironment. Detection of these proteins may identify a tumour where the influences of the tumour microenvironment have enabled a tighter assimilation with the local tissues than others, with this integration enabling some tumour cells to persist or survive within the residual tumour bed after surgery. Reasons to explain why variable levels of VEGF and/or decorin could contribute to the different rates of recurrence or patient survival observed in the current study are discussed below. These include 1) the wider diffusion of tumour cells beyond the gross boundary of the STS and 2) the development of cellular dormancy.

Wider infiltration of tumour cells: One reason to explain why a tumour may recur after resection of the grossly visible mass is that tumour cells have migrated into the peripheral tissues and escape surgical extirpation. The persistence of microscopic clusters of tumour cells within the wound bed is a commonly recognised cause for tumour recurrence. In human STS, microscopic tumour nodules have been identified between 1 cm and 4 cm from the main mass in 30% of cases, with recurrence almost 7-times more

common when these microscopic tumour foci were observed.[67] Similar findings have been reported for other types of tumour.[1, 67-69] In dogs, satellite lesions have been observed in 6% of low-grade STS, with tumour cells extending between 2-17mm from the tumour boundary.[70]

The impact of varying levels of VEGF and decorin identified in STS in the current study may reflect their influence on tumour cells being able to infiltrate beyond the main tumour mass. Studies of human STS have shown that high expression of VEGF receptor-3 (VEGFR-3) is an independent prognostic indicator of reduced disease-free survival in human patients with STS, even when the tumour has been widely resected with clean histologic margins.[57] Because the VEGFR-3 pathway is principally associated with increased lymphangiogenesis rather than angiogenesis,[57] it is possible that tumours with high VEGFR-3 have increased development of lymphatic pathways that enable the increased distribution of cells beyond the local tumour site. In addition, abundant expression of decorin is thought to lead to a more organised ECM, with the strong stromal matrix creating a physical barrier against tumour cell metastasis.[34] Conversely, low levels of decorin in a tumour are thought to create a more fragile matrix structure that facilitates cell migration, which would enable increased local invasion and metastasis.[36] This evidence raises the possibility that the increased rates of recurrence and reduced survival observed in STS with high VEGF and low decorin levels in the current study could be due to a wider distribution of satellite cells radiating beyond the immediate circumference of the tumour. If there is an increased proportion of microscopic satellite lesions persisting in

the wound bed after surgery, this may contribute to an increased rate of recurrence or metastasis after surgery.

Persistence of dormant tumour cells: Dormancy is an innate ability within all living cells that enables survival in the face of unfavourable environmental conditions, particularly hypoxia. Diffusion of oxygen from the blood supply is limited to a distance of between 100 to 200μm; therefore, as a tumour increases in size the expanding cluster of cells will become more and more isolated from the innate blood supply. For a cancer cell to survive in this increasingly hypoxic environment, it must either drive angiogenesis to increase the delivery of oxygen and nutrients to the growing tumour, or adapt its metabolism to allow continued survival in the suboptimal conditions.[71] One of the most potent stimulators for VEGF activity in the tissues is local hypoxia.[62, 64] In Chapter 3, it was shown that larger STS, and tumours with higher levels of necrosis were associated with shorter disease-free intervals and survival times. It is therefore possible that the high VEGF detected in STS in the current study is simply a surrogate indicator for a tumour that has been more hypoxic during its development. As discussed in Chapter 3, hypoxia will develop within a growing tumour if it is unable to develop the necessary vascular supply to support its continued expansion. Across the entire tumour mass, there will be heterogeneity with some areas attaining adequate vascularisation, and other areas where oxygen and nutrient delivery remains poor. In a hypoxic environment, generation of hypoxia inducible factor (HIF-1a) within the affected cell will drive metabolism towards anaerobic glycolysis, with increased production of lactic acid.[72, 73] It has been shown that cells derived from a persistently acidic

environment are often in a dormant state, with cell proliferation held in G0 phase by reduced CDK-1 activity. Such dormant cells are known to be more immune to destruction by chemotherapy and radiation therapy. Thus, while a persistently hypoxic tumour microenvironment will undoubtedly lead to a high percentage of cellular death and tumour necrosis in sections of the tumour, it will also favour a genotype that is more resistant to immune destruction and more capable of surviving in a dormant state. This evidence suggests that the increased rate of recurrence in tumours with high VEGF may be because these STS harbour a higher concentration of dormant cells within the peri-tumoural environment. These dormant cells may survive within the tumour bed following surgical resection, becoming reactivated to grow once environmental conditions improve following resection and tissue healing.[74, 75]

There are several limitations to the presently reported study. One important limitation is that the immunostaining was only interpreted by two people. For a prognostic scheme to be successful in the general population, the assessment criteria for a tumour has to be well-enough defined to minimise inter-pathologist variability. While the immunostaining in the current study appeared easy to assess, additional studies using a larger number of pathologists in different settings is required to ensure consistency in interpretation. Variations in the interpretation of immunostaining intensity may occur as a result of differences in tissue fixation, the immunohistochemical protocol and from intra- and/or inter-observer variability.[76] However, in the current study the assessment criteria relied more on the proportion of positive staining cells, or the distribution within

the tumour. The inter-observer reliability of this more quantitative estimate is considered good, with one study suggesting that pathologists can estimate differences in proportions of objects in an image even if the difference is as little as 5%.[77]

Immunohistochemical investigation of VEGF has previously been performed in a variety of canine and human tumour types.[8, 14, 21, 23, 24, 26, 27, 50, 78-81] Two studies have evaluated VEGF immunostaining in soft tissue sarcoma and fibrosarcoma;[30, 32] while these latter studies did not correlate staining patterns with prognosis or other tumour characteristics, they provide some validation of the immunohistochemical protocol and staining patterns for canine mesenchymal tumour cells. In all previous studies, immunostaining of VEGF has demonstrated a cytoplasmic or perinuclear localisation of the protein, consistent with the observations in the current study. One canine study has described variations in the cytoplasmic distribution and granule size between benign and malignant canine mammary tumours,[81] but this variation has not been reported by other authors.[27, 30, 32, 48]

An additional limitation of the current study was the potential for uncertainty in the quality of follow-up information obtained for all patients, given that this information was obtained retrospectively. The extent of resection performed by the surgeon was based on recall from medical notes written on a surgery performed many years previously. Evidence for tumour recurrence was dependent on the owners returning to their veterinarian; the cause of death was also open to interpretation as almost all dogs were euthanased,

with no postmortem. Also, because this archive set was derived from cases managed in first opinion practice, there was a higher proportion of low-grade STS. The combination of these features may introduce several confounding factors to the results. Validation of this study using multiple pathologists assessing a wider population of STS would be of value. Finally, the number of cases examined in this study was small. Due to selection bias, these results may not be a generalizable to a larger population, so require further validation. The small number of patients also led to some erroneous findings in subgroup analysis. As an example, this study found that almost all STS resected by marginal excision had a high VEGF. This would appear to be a random finding, as there is no valid explanation for why VEGF immunostaining should be influenced by the resection margin. There was no apparent statistical association found between resection margins and outcome of local recurrence or survival in this study. Nevertheless, the influence of this apparent selection bias on the overall results of the study are difficult to predict.

4.5 Conclusion

In conclusion, the results of this study suggest that evaluation of VEGF and decorin levels within a STS may provide information about the biologic behavior of the tumour and allow identification of a tumour that has a higher risk of local recurrence after surgery, and/or to cause the death of the dog due to metastasis. Validation of these results using a wider population of pathologists and patients will be important.

In order to better understand the relationship between VEGF and STS growth, determination of the mRNA expression of VEGF using polymerase chain reaction (PCR) assay was undertaken in the next chapter. The PCR allows a more detailed analysis of gene expression. To the authors knowledge, there have been no studies demonstrating the concordance between IHC and PCR for VEGF in canine STS.

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Chapter 5

Development of a PCR assay to investigate the expression of splice variants of vascular endothelial growth factor in soft tissue sarcomas

5.1 Introduction

In the previous chapter, different patterns of vascular endothelial growth factor (VEGF) and decorin immunostaining were associated with varied rates of local tumour recurrence and overall survival following surgical removal of a soft tissue sarcoma (STS). The conclusion from that study was that identification of these biomarkers in STS would more accurately predict prognosis and could be used to guide clinical decisions. For example, these biomarkers could potentially be used to identify dogs that are more likely to benefit from active monitoring or adjuvant therapy to help detect or prevent recurrence after surgery.

In the previous study, quantification of these proteins was performed using immunohistochemistry (IHC). While IHC is recognised as an economical and rapid method for examining tumour tissue for the status of a biomarker.[1, 2] the results can be difficult to interpret with often quite significant variability between pathologists and between laboratories.[2] Additionally, there can be variation in the intensity of protein immunostaining in different sections of the tumour due to heterogeneity within the tissues. This may lead to further

variability in the interpretation of the IHC result, particularly if one section of the tumour contains more intense immunostaining than other sections from the same neoplasm.[2] An alternative method for evaluating biomarkers in tissues is to quantify the expression of the DNA for the biomarker. This can be done by measuring mRNA using the reverse transcriptase polymerase chain reaction (RT-PCR).[3] In contrast to IHC, RT-PCR is mostly an automated method that produces an objective value. Therefore RT-PCR will not be influenced by the inter-observation variation that is unavoidable with subjective assessment of immunostaining.

Another important distinction between IHC and RT-PCR is that IHC simply detects the presence of a certain protein within the tumour.[1] By comparison, RT-PCR is able to measure the quantity of RNA within the tissue.[3] This distinction is important because the quantity of RNA does not always correlate with the quantity of protein that is produced.[4] The development of this difference between the amount of gene expression and the quantity of protein produced by a cell can be a significant process in the neoplastic transformation of a cell. Differences can occur as a result of post-transcriptional or post-translational defects leading to low protein production despite large quantities of mRNA being produced. Alternatively, if there is lower gene expression than normal, as well as reduced quantities of the relevant protein within a cell, this may suggest that gene expression has been silenced by mutation or through methylation of the gene promotor. Furthermore, the presence of large quantities of protein but little RNA suggests the presence of large quantities of altered protein that cannot be broken down by the neoplastic cell.

One of the mechanisms that may result in a discrepancy between gene expression and protein production is alternative RNA splicing.[5] Splicing is a precisely regulated process that occurs after gene transcription, but before mRNA translation. Splicing occurs as a result of deletion or re-arrangement of different portions of the pre-mRNA molecule, with 'cut and paste' reactions between different intron and exon boundaries being catalysed by a small spliceosome enzyme. Rearrangement of the pre-mRNA molecule leads to the production of a variety of different mature mRNAs from a single gene; this process provides some explanation for the complexity and diversity of protein morphology that occurs in mammals.[5] Different splice variants can have different physiological activities due to loss or addition of functional domains. For example, it has been reported that some splice variants may actually block the normal function of a protein, perhaps providing some feedback control of ligand activity. Some splice events can also transform membrane-bound proteins into soluble proteins, allowing them to have a wider influence within the tissues.

The impact of alternative splicing on the VEGF molecule has been described in humans,[6] and the dog.[7] The VEGF gene typically consists of eight exons,[8] and all of the VEGF isoforms share a highly preserved VEGF homology domain, encoded by exons 1 to 5. Exons 6 and 7 encode for two distinct heparin-binding domains, and the expression of these varies between different isoforms. In people, at least 9 different isoforms have been described: these have been termed VEGF206, 189, 183, 165, 148, 121 and 111.[6] Each isoform shows variability both in their localisation within the tissues as well as their physiological activity, as described below:

- VEGF206, 189, 183, 162 and 145 tend to be tightly bound to heparin-containing proteoglycans in the extracellular matrix (ECM), such as decorin. The bioavailability of these forms is dependent on release by heparinase or other proteolytic enzymes, and their activity is less potent. The ECM largely acts a repository for these isoforms, where they can exert their effect over a longer period of time.
- VEGF121 and 111 are highly soluble isoforms and have potent angiogenic properties. They are freely diffusible within the tissues.
- VEGF165 has angiogenic properties that are intermediate to the isoforms described above. It retains heparin-binding ability within the ECM, but about 50% of the protein remains cell-associated.

Mutations within a neoplastic cell can disrupt alternative splicing, resulting in increased or decreased production of specific protein isoforms.[5] Some authors suggest that this altered production of protein isoforms can influence the initiation or progression of the cancer.[9] In humans, VEGF121 expression has been shown to be increased relative to VEGF165 in colorectal, prostate and breast cancer, and this increase correlates with enhanced angiogenesis within the tumour.[9-12] In human osteosarcomas, VEGF165 expression was significantly correlated with the development of metastases ($P = 0.005$); in one study, patients with an osteosarcoma that did not express the VEGF165 isoform had a significantly improved overall survival compared to patients with osteosarcomas that did express this isoform.[13] Other studies of human cancers have shown that not only do different tumour types express varying isoform profiles, but other angiogenic factors or modulators

within the tumour environment influence whether the soluble or membrane-bound VEGF isoforms are more likely to have a dominant role.[14]

In the dog, five VEGF isoforms have been described.[7, 15] These isoforms are almost identical in structure to the human molecules apart from a single glutamic acid residue which is missing at position 5 of the canine VEGF protein. This deletion has also been noted in VEGF from other mammalian species and is not thought to influence the biological activity of the protein.[7] Due to the missing glutamic acid the canine isoforms are designated VEGF188, 182, 164, 144 and 120. It is presumed that the physiologic activity and tissue binding properties of the canine isoforms are similar to those described for human isoforms, with VEGF120 being the most soluble form, and VEGF188, 182 and 144 being tightly bound to proteins in the ECM.

To date, there have been few investigations into the expression of the different VEGF isoforms in canine cancer and no study has evaluated whether or not different isoforms have prognostic significance.[7] One study investigated the expression of three VEGF isoforms (VEGF120, VEGF164, VEGF188) in canine primary central nervous system tumours.[15] All three isoforms were detected in all of the tumour types examined, with VEGF164 being the predominant isoform in grade 3 oligodendrogliomas. However, no attempt was made to correlate prognosis with variations in VEGF mRNA expression in that study.

To the author's knowledge, there have been no studies comparing VEGF immunostaining and VEGF expression in canine STS. Additionally, the

presence of different VEGF isoforms within a STS has never been evaluated and no studies in veterinary medicine have associated the VEGF isoforms in a neoplasm to the subsequent clinical behaviour of that neoplasm. Therefore, the aim of this study was to measure the expression of VEGF isoforms within a series of canine STS with known VEGF immunostaining and known recurrence and survival times. The hypothesis of this study was that there would be an overall increase in VEGF expression in tumours with increased rates of local recurrence or reduced survival time, in concordance with the results of the IHC study. Additionally, tumours with increased rates of local recurrence or reduced survival time would show a different ratio of expression of individual VEGF isoforms than tumours where no recurrence or tumour-related death occurred after surgery.

5.2 Materials and method

5.2.1 Sample selection

Samples included those in the tissue archive of 350 STS that had been established in Chapter 3. Clinical details about each STS had been determined by questionnaire, as detailed previously.

For the current study, cases were excluded if the status of any of the following criteria was recorded as unknown: tumour recurrence, tumour as a cause of death, tumour grade, tumour size, location, palpable characteristics, and the degree of resection. Cases were also excluded if the STS had been removed by amputation. This left a total of 136 cases. A total of 71 samples were randomly selected from this final cohort using the CRAN package "sampling" in R (R

version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria). Stratification was performed to provide an equal allocation of histological grades across the main outcome measure of local tumour recurrence.

To maximize the chance of having sufficient tissue for RNA extraction, formalin-fixed paraffin-embedded (FFPE) blocks were examined from each dog included in the study. For inclusion, tissue blocks had to contain a large piece of tissue in which the tumour occupied over 50% of the tissue. If no suitable FFPE blocks were available for an individual dog, then another case was randomly selected from the total population pool. This new dog had to have a STS of the same grade and tumour recurrence status as the excluded dog.

5.2.2 Cloning positive controls

Positive controls for VEGF were developed by cloning DNA fragments designed from the three VEGF splice variants (NM_001003175.2, NM_001110501.1, NM_001110502.1, known as VEGF splice variant 1, 2 & 3 respectively) that have been categorised on the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/nuccore/?term=Canis+lupus+familiaris+VEGFA). Sequence-verified, double-stranded DNA Gene Fragments (gBlocks) were obtained from Integrated DNA Technologies (Coralville, IA, USA). A further positive control for the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used in this study was cloned from genomic DNA extracted previously. Primers used to produce the GAPDH

PCR product were based on those published previously.[16] A PCR was performed to amplify the GAPDH fragment as follows: 1x FIREPol master mix (Solis Biodyne, Estonia), 250nM of each primer, 50ng of template DNA, and then made up to a total volume of 20µl with nuclease free water. Thermal cycling conditions were: 95°C for 15 min, followed by 40 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, with a final extension at 72°C for 7 minutes. PCR products were separated via electrophoresis (1% w/v agarose (Bioline, UK) in 0.5x TBE), products of the appropriate size were excised and eluted overnight at 4°C in elution buffer (10mM Tris-HCl). The PCR product from the gBlocks and GAPDH were cloned into *E. coli* using the Invitrogen TOPO TA Cloning Kit with One Shot TOP 10 cells following the manufacturer's instructions. (K45750, ThermoFisher Scientific, USA). Plasmids containing the cloned gBlocks and GAPDH were then extracted from the bacterial colonies using Invitrogen PureLink Quick Plasmid Miniprep extraction kit following the manufacturer's instructions (K210011, ThermoFisher Scientific, USA). The plasmid DNA was stored at −20°C until used in further experimental methods.

Confirmation of successful cloning was performed using M13 insert flanking primers to amplify all clones.[17] The PCR to amplify the clones was performed as follows: 1x FIREPol master mix (Solis Biodyne, Estonia), 250nM of each primer, 50ng of template DNA, and then made up to a total volume of 20µl with nuclease free water. Thermal cycling conditions were: 95°C for 15 min, followed by 40 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, with a final extension at 72°C for 7 minutes. PCR products were separated via electrophoresis (1% w/v agarose (Bioline, UK) in 0.5x

TBE). Products of the appropriate size were excised and eluted overnight at 4°C in elution buffer (10mM Tris-HCl). Eluted PCR product was sent to Massey Genome Service (Massey University, New Zealand) for bi-directional Sanger sequencing. Results were compared to the target sequences using Geneious (v. R8.1) (https://www.geneious.com, Biomatters, Ltd., Auckland, New Zealand).

5.2.3 Designing and testing primers

Primers to amplify three of the currently recognised splice variants of canine VEGF were designed using the Geneious software (version R8.1) (https://www.geneious.com, Biomatters, Ltd., Auckland, New Zealand). This included VEGF188 (AF133250.1), VEGF182 (AF133249.1), and VEGF164 (AF133248.1). Primers were also developed to amplify the mRNA specific for the homologous region of VEGF. Three different primers for this portion of the VEGF gene were developed to try and create a shorter PCR amplicon product which would reduce the time of the program. Primers were designed to amplify a 75-200bp product with a GC content that was 50-60%, with G and C repeats that were no longer than 3 bases. Regions with long repeats of single bases (>4) were avoided. Details of the primers selected are shown in Table 5.1.

All primers were tested using the cloned VEGF spliced variants as templates, initially via endpoint PCR. Endpoint PCR was performed as follows: 1x FIREPol master mix (Solis Biodyne, Estonia), 250nM of each primer, 50ng of template DNA, and then made up to a total volume of 20µl with nuclease free

Table 5.1

Details of the primers designed to amplify the individual VEGF splice variants, the homologous region of total VEGF and the housekeeping gene GAPDH

Locus	Primer details	Forward primer (5' – 3')	Reverse primer (5' – 3')	Annealing Temp endpoint	Annealing Temp qPCR
VEGF	Splice variant 1: VEGF 188	GTA TAA ACC CTG GAG CGT TC	TTT AAC TCA AGC TGC CTC GC	58°C	N/A
	Splice variant 2: VEGF 182	GAA AGC GCA AGA AAT CCC GTC	TTT AAC TCA AGC TGC CTC GC	58°C	N/A
	Splice variant 3: VEGF 164	GAT AGA GCA AGG CAA GAA AAT C	TTT AAC TCA AGC TGC CTC GC	58°C	N/A
	Total VEGF-ver1	CAA CAT CAC CAT GCA GAT TAT G	ACA CGT CTG CGG ATC TTG TAC	52°C	N/A
	Total VEGF-ver2	CAT AGC AAA TGT GAA TGC AGA C	ACA CGT CTG CGG ATC TTG TAC	52°C	60°C
	Total VEGF-ver3	CAA CAT CAC CAT GCA GAT TAT G	GTA CAA GAT CCG CAG ACG TGT	52°C	60°C
GAPDH	GAPDH	GGA GAA AGC TGC CAA ATA TG	ACC AGG AAA TGA GCT TGA CA	52°C	60°C

water. Thermal cycling conditions were: 95°C for 15 min, followed by 40 cycles of 95°C for 30 sec, annealing for 30 sec, 72°C for 30 sec, with a final extension at 72°C for 7 minutes. Annealing temperatures varied by primers and ranged between 50°C to 60°C. The PCR products were separated via electrophoresis and sent for Sanger sequencing when necessary, using the same protocol as described previously.

5.2.4 Primer Validation

The primers designed to amplify the splice variants were amplified via quantitative PCR (qPCR) under the following conditions: 1x KAPA SYBR

FAST qPCR Master Mix (MS, USA), 0.2μM of each primer, 2μL of template plasmid DNA and nuclease free water to create a total volume of 20μL. All RT-qPCR reactions were performed in a Qiagen RotorGene Thermal cycler (Hilden, Germany) under the following conditions; 95°C for 3min, 45 repeats of 95°C for 10sec and 60°C for 30sec and 72°C for 30 secs, with melt analysis from 72°C to 95°C in 0.2°C/sec increments.

5.2.5 Extraction of RNA from soft tissue sarcoma samples

Total RNA was extracted from the FFPE samples selected previously using the High Pure FFPE RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Briefly, tissue sections were cut at a thickness of 10μm and deparaffinised in xylene and then graded ethanol (absolute and 70%). A stepwise tissue lysis step using proteinase K was performed until no particulate matter remained. This was followed by RNA isolation on a filter column through a series of wash buffer rinses. The RNA was finally eluted into a collection tube using elution buffer.

Samples were stored at -80°C until required. RT-qPCR was then performed on each sample, using the protocol as described in subsequent sections.

5.2.6 Reverse Transcriptase Quantitative PCR assays

Reverse transcriptase quantitative PCR of the VEGF and GAPDH primer set was performed on all extracted RNA in triplicate. Included in each PCR run was a serial dilution of the appropriate plasmid ranging from 0.2 - 0.0000002 ng/μl (100pg – 0.1 fg/μl). All RT-qPCR were performed using the

KAPA SYBR FAST One-Step qRT-PCR Kit according to the manufacturer's specification. Briefly, into each 0.2mL thin-walled PCR tube on ice was added 1x KAPA SYBR FAST qPCR Master Mix (MS, USA), 0.2µM of each primer, 2µL of template mRNA, 1X KAPA RT Mix and 7.2µL nuclease free water to create a total volume of 20µL. All RT-qPCR reactions were performed in a Qiagen RotorGene Thermal cycler (Hilden, Germany) under the following conditions; 42°C for 10 min, 95°C for 3min, 45 repeats of 95°C for 10sec and 60°C for 30sec and 72°C for 30 secs, with melt analysis from 72°C to 95°C in 0.2°C/sec increments. A negative control containing nuclease free water was used in all assays. Melt curve predictions were determined using uMel;[18] the expected melt temperature was predicted as 88.5°C for the VEGF primer set and 92.5°C for the GAPDH primer set.

5.3 Results

5.3.1 Primer validation

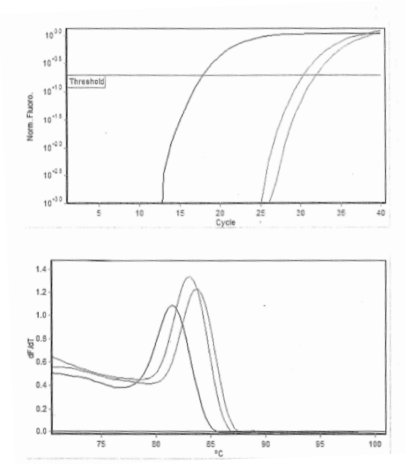
Primers for splice variants 1 and 2 were both successfully amplified and sequenced. However, efforts to amplify splice variant 3 proved problematic, despite modifications to conditions. After repeated efforts, the results from agarose gel electrophoresis indicated that the combination of forward primer for splice variants 1, 2 and 3 with the reverse primer for Total VEGF-ver3 produced the most consistent results. These primers were then tested using quantitative RT-qPCR.

5.3.2 Reverse Transcriptase and Quantitative PCR of the splice variants

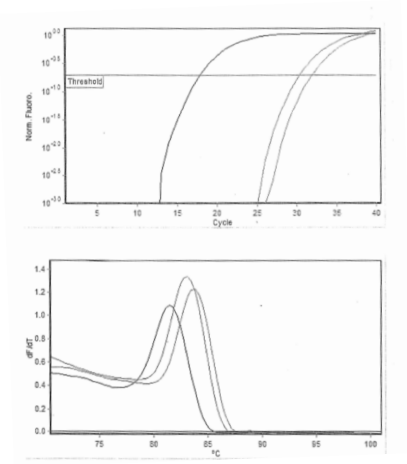
Even though there were significant differences in genetic sequences between the three splice variants, by coincidence all melt temperatures were identical to each other (Figure 5.1). Despite modifications to the qPCR parameters and primer concentrations, it was not possible to quantify individual splice variants with this protocol. The study was continued but instead of trying to examine individual VEGF isoforms, only total VEGF was evaluated.

Figure 5.1:
Amplification and melt curves from RT-qPCR of each strain variant.

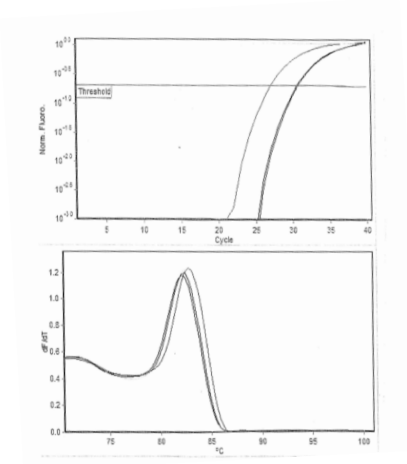
Splice variant 1: VEGF 188



Splice variant 2: VEGF 182



Splice variant 3: VEGF 164

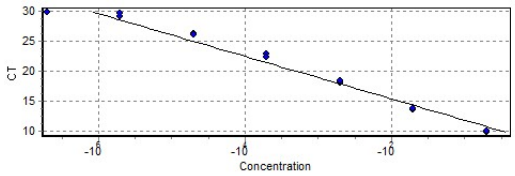


5.3.3 Primer validation: Standard curve for VEGF and GAPDH

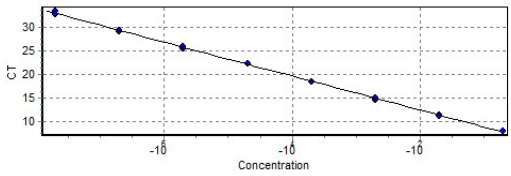
The results of amplifications using primer Total VEGF-ver3 and housekeeping gene GADPH at serial dilutions are shown in Table 5.2. For VEGF, amplification had a correlation coefficient (R^2) of $R^2 = 0.97066$. The melt curve showed a single inflection point, suggesting a pure amplicon product was produced (Figure 5.2a). For the GAPDH reference gene, the amplification standard curve showing the Ct at reducing concentrations of starting product had an R^2 of 0.99924. Melt curve temperatures had a single inflection point, confirming a pure amplicon product (Figure 5.2b).

Table 5.2:
Standard curve qPCR values for VEGF (left) and GAPDH (right) primers

VEGF



GAPDH



VEGF					GAPDH			
Name	Given Conc (ng/ul)	Ct	Melt Temp (°C)		Name	Given Conc (ng/ul)	Ct	Melt Temp (°C)
100pg	0.2	9.79	84		100pg	0.2	9.79	84
10pg	0.02	13.52	84		10pg	0.02	13.52	84
1pg	0.002	18.07	84		1pg	0.002	18.07	84
100fg	0.0002	22.87	84		100fg	0.0002	22.87	84
10fg	0.00002	26.13	84		10fg	0.00002	26.13	84
1fg	0.000002	29.11	84		1fg	0.000002	29.11	84
0.1fg	0.0000002	29.67	84		0.1fg	0.0000002	29.67	84
0.01 fg	0.00000002	33.4	84		0.01 fg	0.00000002	33.4	84

Figure 5.2a and 5.2b

Amplification curves (left) and melt curves (right) arising from serial dilutions of total VEGF and GAPDH.

Figure 5.2a: Vascular endothelial growth factor (VEGF)

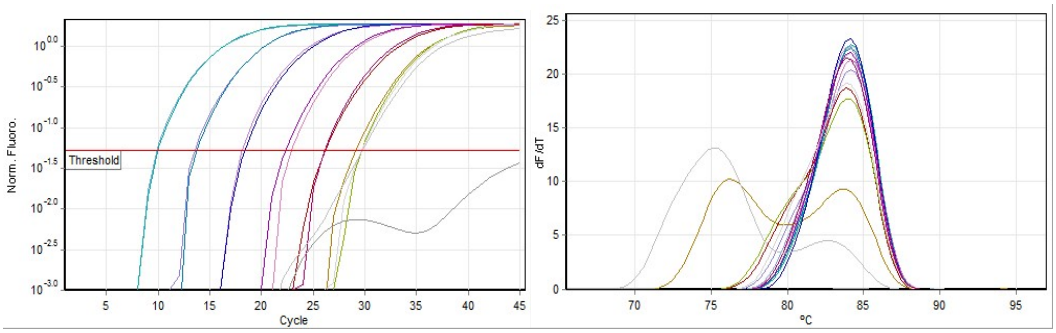
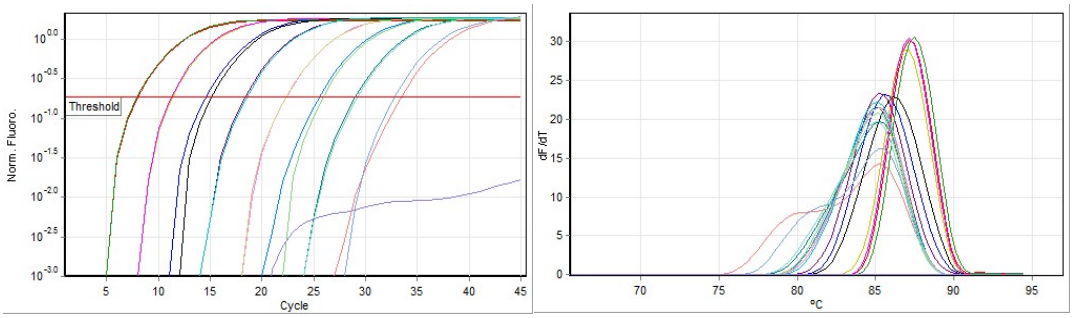


Figure 5.2b: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)



5.3.4 Measurement of RNA extraction from FFPE blocks:

RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer on twelve samples only. RNA concentration in these samples was considered good, ranging from 17.4 – 1059 ng/µL (Table 5.3). These results provided confidence in the sample extraction methodology. Due to time constraints, measurement of RNA concentration in the remaining samples was not performed.

Table 5.3:
RNA concentrations from the subset of soft tissue sarcoma samples, as measured by Nanodrop ND-1000 spectrophotometer

Sample No.	**RNA concentration (ng/ul)**
1	17.4
2	122.4
3	476.8
4	407.4
5	50.7
6	645.4
7	125.2
8	18.4
9	430.2
10	91.8
11	1054.4
12	100.2

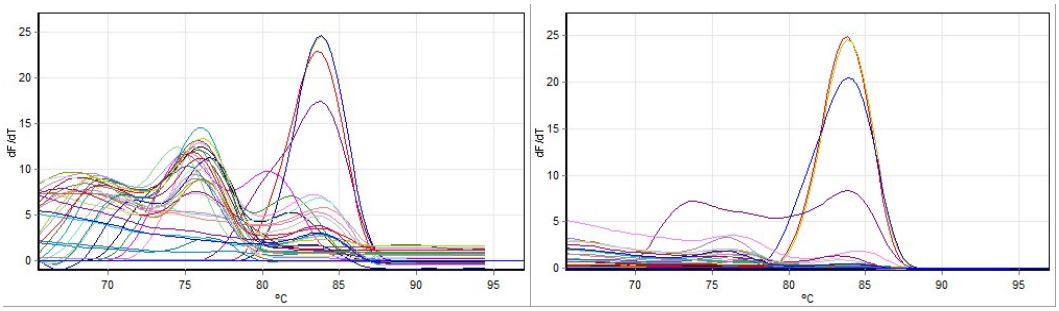
5.3.5 Reverse Transcriptase and Quantitative PCR (RT-qPCR): VEGF expression in STS samples

Reverse transcriptase and quantitative PCR was performed in triplicate on 27 individual STS cases, so a total of 81 reactions were performed. The R^2 value of the standard curve for the VEGF template in each individual RT-qPCR run had values ranging from 0.98486 to 0.99755 confirming successful amplification conditions.

By comparison, Ct values for the STS samples were poor. In 18 of 81 RT-qPCR reactions, no amplicon product was detected. In the remaining 63 reactions, the Ct ranged from 30.27 to 43.78. However, in these cases, multiple melt temperatures were observed suggesting a heterogenous PCR product was present. In both cases, a single inflection peak was observed for

the VEGF template, suggesting the PCR conditions were appropriate (Figure 5.3a and 5.3b).

Figure 5.3a and 5.3b
Examples of melt curves from the RT-qPCR assays performed on two different cohorts of STS samples. Multiple melt temperatures are observed for most sample reactions. A single inflection peak was observed for the VEGF template, suggesting good PCR conditions were present.

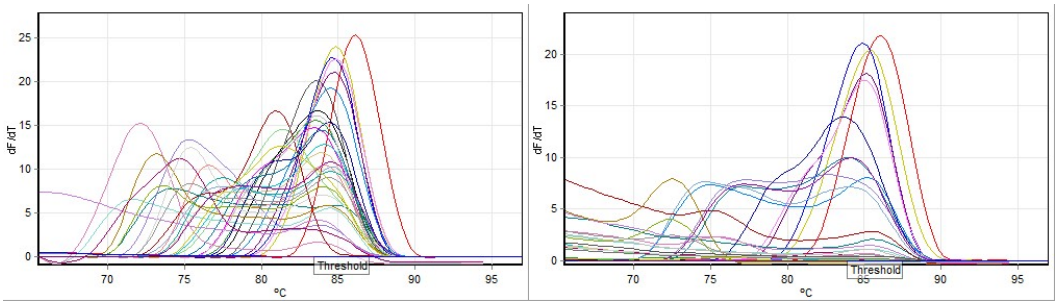


5.3.6 Reverse Transcriptase and Quantitative PCR (RT-qPCR): GAPDH expression in STS samples

Reverse transcriptase and quantitative PCR for the house-keeping gene GAPDH was performed in triplicate on 18 individual STS cases, giving a total of 54 reactions. The R^2 value of the standard curve for the GAPDH template in each individual RT-qPCR run had values ranging from 0.98486 to 0.99755 confirming successful amplification conditions.

By comparison, Ct values for the STS samples were poor. No amplicon product was detected in 13 of 54 reactions. In the remaining 41 reactions, the Ct ranged from 28.5 to 44.12. Once again, multiple melt curve temperatures were observed (Figure 5.4a and 5.4b).

Figure 5.4a and 5.4b
Examples of melt curves from the RT-qPCR assays performed on two different cohorts of STS samples. Multiple melt temperatures are observed for most sample reactions. A single inflection peak was observed for the GAPDH template, suggesting good PCR conditions were present.



5.3.7 Further analysis abandoned

Because of the poor performance of the PCR assay with both the VEGF and GAPDH reference gene primers, the study was paused to allow for further validation. However, due to inadequate quantities of necessary reagents, no further RNA could be extracted from the FFPE blocks to support additional investigations. The study therefore had to be abandoned at this point.

5.4 Discussion

Polymerase chain reaction is a relatively routine technique, so it is frustrating when it does not work as planned. However, there are many components which can disrupt the amplification process. In the current study, two major problems occurred that prevented successful achievement of the research goals. Firstly, there was inconsistent amplification when using both the VEGF and GAPDH primers on mRNA extracted from the soft tissue sarcoma

samples. Secondly, the PCR reaction could not be validated to allow quantification of the RNA for each of the VEGF splice variants.

The inability to amplify VEGF or GAPDH from any STS sample in the study was considered most likely to be due to an inadequate quantity of amplifiable RNA within the sample. This interpretation was based on the amplification and melt curves derived in the study. The amplification curves from the RT-qPCR revealed that either no or inadequate amounts of RNA was being amplified from the mRNA extracted from the STS samples. The amplification curve provides a visualisation of the gene products being produced in the PCR assay, by measuring the magnitude of fluorescence within the amplicon.[3, 19] In the current study, the PCR assay made use of a fluorescent dye, SYBR green. SYBR green will bind avidly to double-stranded DNA (dsDNA), with binding causing the magnitude of its fluorescence to increase.[3] The cycle threshold (Ct) is defined as the number of cycles required before the fluorescence emanating from dsDNA exceeds the threshold of background noise. Measurement of Ct therefore gives a real-time indicator of the quantity of nucleic acid that has developed within the sample. The RT-PCR protocol used in this study underwent 45 cycles of amplification. Under ideal conditions, the RT-PCR reaction should have amplified the initial quantity of target RNA for fluorescence to be detectable within 29 cycles, giving a Ct of ≤29.[19] This reading is indicative of a strong positive reaction and suggests there should be abundant target nucleic acid in the sample. A Ct of between 29-38 would be considered weakly positive, suggesting there were only moderate amounts of nucleic acid present in the PCR vial. Readings over this level are considered only weakly positive and are

likely unreliable for analysis. In the current study, the Ct was unmeasurable in 18 out of 81 reactions with the VEGF primer, and in 6 out of 47 reactions with the GAPDH primer. When PCR product was detected in the remaining reactions, the Ct for VEGF ranged from 30 to 43 (mean Ct = 37.3) and 28.5 to 44 (mean Ct = 36.7) for GAPDH. In each PCR assay, the Ct for the positive control templates for both VEGF and GAPDH increased predictably in accordance with the serial dilution of the starting product. The R^2 of the standard curve from each PCR experiment was consistently greater than 0.99, providing confidence that the cycling conditions and all reaction solutions that were used for the qPCR were stable and correct.[19]

The positive Ct readings generated from some of the samples in the current study may suggest that copies of the target gene had been amplified, raising the prospect for an interpretable result in these cases. However, analysis of the melt curve suggested the PCR product in these cases was probably a heterogenous nucleic acid population, rather than a pure clone of VEGF or GAPDH RNA being amplified.[3, 20] Melt curve analysis is an important diagnostic measure to verify the specificity of the PCR assay, and to determine that the amplicon present at the end of the amplification phase is indeed the target gene.[21] The assumption behind the melting curve is that the amplicon will be homogenous and will therefore degenerate spontaneously at identical conditions. If the amplicon is a single gene product, a single large melting peak will be observed in the melt curve when 50% of the dsDNA has degenerated. However, if amplification has resulted in multiple gene fragments, then multiple melt peaks will be obtained. In the current study, melt curves from reactions using the VEGF and GAPDH

primers with the STS samples were composed of multiple distinct peaks. This suggests that non-specific amplification had resulted in non-target RNA being amplified. The melt curves from each of the PCR assays did have distinct single inflection peaks that related to degeneration of the amplicon generated from the positive control VEGF template. The presence of these peaks confirmed that amplification of the positive control template had performed as expected.

The interpretation of these findings is that no significant quantities of VEGF or GAPDH RNA was being amplified from the majority of STS samples during RT-PCR. In the few samples from which RNA was amplified, this appeared to be multiple gene sequences, suggesting that very little total RNA had been extracted from the STS samples.

In the current study, the concentration of extracted RNA was only measured in 12 of the 27 samples due to time-constraints, with a focus on generating reportable data from the experiment. The decision to not measure extracted RNA in all samples was influenced by the detection of adequate concentrations of RNA extracted from the 12 STS samples where analysis was performed. Ideally, determination of RNA concentration should have been performed on all samples to provide confidence that adequate RNA was available for amplification in every case.

Nevertheless, even if the concentration of RNA was adequate, another potential problem in the current study is the quality of the RNA was not determined. While RNA is a relatively stable biological protein, it is prone to denature when exposed to unfavourable conditions.[22, 23] There are a

number of factors that could have reduced the quality of the RNA in this study as the sections of STS used in this study were initially fixed in formalin following surgery and then stored as FFPE blocks for more than 13 years. Processing conditions for the samples were directed more towards the establishment of a histological diagnosis for clinical reasons, rather than the precise preservation of the tissue for molecular research. For this reason, the processing and storage conditions that the tissues were exposed to will have created a number of unfavourable factors that could have influenced the integrity of the RNA within the sample.

Two of the major factors known to affect the quality of the RNA within FFPE samples is the time spent in fixative solution prior to processing and the age of the block.[23, 24] Fixation in formaldehyde for periods of between 48 hours and 7 days, or longer, has been shown to have a detrimental effect on the amplification efficiency of RT-PCR.[22, 23] In the current study, it was impossible to know the time the samples would have spent in formaldehyde prior to being processed and embedded into paraffin. The samples had been obtained from a commercial laboratory that serves the veterinary community in the UK. Under most instances, it could be presumed that the STS specimens will only have been stored in formaldehyde for the time taken to be transported by a postal or courier service from the clinic to the laboratory, a period that would likely range from 24-48 hours for samples sent on a weekday. However, this duration could increase to 72 hours or more for samples where transportation to the laboratory was disrupted by weekends, holiday periods or other logistical delays.

The size of the tissue samples being fixed will also impact on the quality of the RNA extracted;[22] if a tissue section is thicker than 2 cm, penetration of formaldehyde into the deeper portions of the tissue will be delayed. Cells deep within the tissue may start to autolyse and degenerate due to the lack of oxygen and nutrients. In the current study, 40% of the tumours were described as being more than 5cm in diameter. This would result in a surgical specimen that was at least 7-8 cm and up to 11-12 cm if a circumferential surgical margin of between 1-3 cm was obtained. It is therefore possible that some tissues will have arrived at the laboratory being inadequately fixed, with subsequent autolytic changes affecting the integrity of the tissue. While these failings may not have had a profound impact on the ability to provide a reliable histological interpretation, there will be potential consequences on the integrity of the nucleic acid required for the RT-PCR analysis in the current study.

Another factor that will influence RNA quality was the storage conditions of the FFPE blocks since their creation.[22, 23] It has been shown that after 1-year storage at 4°C, ribosomal RNA extracted from tissue will usually still be of acceptable quality.[22] However, other authors have shown that after 4 years of storage at ambient temperatures, RNA quality will deteriorate significantly.[23, 24] In the current study, the FFPE blocks had been stored for almost 13 years prior to being sectioned for the RT-PCR study. During this time, their storage conditions were less than optimal, having been transported half-way around the world, and kept at room temperature with wide fluctuations in range depending on the season. The possibility that

significant RNA degeneration was responsible for the poor RT-PCR results in the current study was therefore high.

Because of the age and poor storage conditions of the FFPE blocks used in the current study, efforts to analyse the quality of RNA extracted from the tissue sections should have been performed, particularly when it became evident that RT-PCR analysis was not working as expected. The most obvious evidence that the quantity or quality of RNA extracted from the samples was inadequate was the consistent failure of the RT-PCR for the house keeping gene GAPDH, which is expected to be consistently expressed in all tissues.

The quality of RNA in a sample is a measure of the degree of degradation due to the effects of various nucleases on the molecular structure.[22] Gradual degradation of RNA will be reflected by a progressive shift towards shorter fragment sizes. Thus, although the total RNA concentration may be acceptable, the RNA strands have become increasingly fragmented which may interfere with amplification during a RT-PCR assay. There are several alternative methods that could have been used to evaluate the quality of the RNA. These include the RNA integrity number (RIN),[25] the paraffin-embedded RNA metric (PERM),[26] and the fragment analysis metric (DV200).[27] One study has suggested there is considerable variation in the usefulness of these different RNA quality measures for FFPE blocks that have been stored for periods ranging from <2yrs under ideal conditions, to >21 years at room temperature.[28] However, in most instances, fragment analysis (DV) outperformed PERM and RIN in determining sequencing quality for gene detection. None of these techniques were employed in the

current study as there was no access to an Agilent bioanalyser required to generate the necessary data for calculation of each metric.

The other problem encountered in the current study was the inability to generate individual PCR amplicons for the VEGF splice variants of interest. Each of the primers were designed using Geneious software (version R8.1) (https://www.geneious.com, Biomatters, Ltd., Auckland, New Zealand), with the intent that each primer would be specific for each splice variant. However, melt curve analysis from the PCR amplicons proved challenging as, by coincidence, all three splice variants had the same melt temperature. Normally, the melt temperature would be used to confirm that the nucleic acid product being generated was consistent with the target gene of interest. Because this was not the case for the VEGF splice variant primers used in the current study, it would only have been possible to determine if the amplicon generated was unique to the primer of interest by performing sequencing analysis.

In the current study, primer design was difficult as there was a lack of significant sequence variation between the different splice variants. This caused a potential overlap between the primers, which could have led to amplification of an incorrect product. To understand this finding, each primer sequence was entered into the nucleotide Basic Local Alignment Search Tool [(BLASTn), National Centre for Biotechnology Information (NCBI), Bethesda, MD, USA] to determine the apparent specificity of the primers to the canine genome. This search showed that the primers designed for VEGF strain variant 1 and VEGF strain variant 2 had good homology with

the mRNA for VEGF188 and VEGF182, respectively. However, the primer for VEGF strain variant 3 showed evidence of alignment with VEGF188, VEGF182 and VEG164. Analysis of the exon structure of the known canine splice variants shows the close similarity between the isoforms chosen for study in this chapter with the only difference between variations in expression of exons 6a and 6b. It is possible that the primer design that was performed at the time was inaccurate. It may also have been preferable to try and study some of the other isoforms such as VEGF 144 or VEGF 120, which may have had a greater variation in sequence structure. At the time this study was being designed, there were limited publications investigating splice variants of VEGF in the dog. In hindsight, using primers published from the previous study on canine VEGF isoforms may have yielded a better result.[15]

The failure of this study to generate interpretable results was frustrating, as it limits our further understanding of the importance of VEGF and, by extension, decorin on the prognosis of STS. At the start of this Chapter, it was hypothesised that there would be different ratios of expression of individual VEGF isoforms between STS that have a higher tendency to recur or cause the death of a dog compared to tumours that have a good prognosis after surgery. One of the goals of this chapter was to try and obtain more insight on the possible interactions between VEGF and other molecules within the tumour microenvironment, such as decorin. In the previous chapter, it was determined that both decorin and VEGF had an influence on STS prognosis. It is hypothesised that decorin may act as a tumour suppressor by sequestering a number of important ligands and growth factors within the

matrix, thereby influencing ligand-receptor interactions and attenuating down-stream signaling pathways.[29, 30] Therefore, a loss of decorin within the microenvironment of a STS, which was shown in the previous Chapter to be associated with reduced disease-free survival, will increase the availability of these ligands and growth factors, which may help drive tumour progression. Because there is a variable degree of binding affinity by VEGF splice variants to matrix proteoglycans such as decorin, it would be important to understand whether the increase in VEGF observed in some STS is due to an increase in the expression in one or all VEGF isoforms, or whether the loss of decorin simply increases the bioavailability of VEGF isoforms that would normally be sequestered in the matrix, without an overall increase in expression.

In the current study, the processing and storage conditions that the tissues were exposed to were presumed to have created a number of unfavourable factors that influenced the integrity of the RNA within the sample. Because utilisation of FFPE samples provides a valuable resource for biomarker and molecular research in oncology, the impact of storage is increasingly recognised.[24] There is a potential for PCR analysis to generate spurious results that are more a reflection of biospecimen handling rather than those of disease state, and false interpretation of these results could therefore confound patient diagnosis. It is generally recommended that any biomarker discovery from FFPE tissue should be validated against fresh frozen tissue to ensure any disparity detected in nucleic acid levels is a true reflection of the disease condition.[22]

The failure of this study means additional investigation will need to be performed to determine if analysis of VEGF could become a reliable prognosis test for STS. If a prognostic test is to be of clinical use, it would need to be simple to perform, reliable and cost-effective to perform. The result should also influence clinical decision making. In addition to the differences between PCR and IHC discussed in the introduction to this Chapter, PCR has the advantage of being able to be performed on very small cellular samples. There are existing analyses currently used in a veterinary clinic for other disease conditions that can be performed on cellular samples obtained by fine needle aspirate. If validated for STS, such a test would lend itself favourably to quantification of the VEGF expression within the tumour. If this test was performed pre-operatively, it could potentially help a clinician decide the optimal resection margins prior to surgery being performed. By comparison, IHC typically requires histological sections of tissue for reliable immunostaining. This tissue would need to be obtained by biopsy and necessitate exposure of the cells to formaldehyde, which is known to have a negative influence on RNA quality. Furthermore, because there is heterogeneity in VEGF expression between different sections of a STS, multiple sections of a tumour would need to be examined to provide a reasonable overview of the tumour, necessitating multiple sections of tumour to be obtained. By comparison, it would be a simple matter for a clinician to obtain multiple fine needle aspirate samples from a variety of sites in the tumour, with these samples being pooled for PCR analysis. Further study will be required to correlate VEGF immunostaining with VEGF expression in canine STS if such a prognostic test was to be developed in the future.

5.5 Conclusion

In conclusion, the aims of this study were not achieved due to technical challenges and failings within the experimental design. It is likely the age and storage conditions of the FFPE blocks required a more sensitive approach to analysis than was originally anticipated. It is possible with more time and resources, viable results could have been obtained.

Nevertheless, there is sufficient evidence from previous studies to suggest that evaluation of VEGF and decorin levels within a STS could allow identification of a tumour that has a higher risk of local recurrence after surgery, and/or to cause the death of the dog due to metastasis. The findings from Chapter 4 suggested that a combination of immunostaining results provided an enhanced ability to identify a small subset of tumours with a reduced risk of recurrence or tumour-related death after surgery. This finding supports a strategy where a suite of different prognostic markers could be used to better predict individual tumour behavior, rather than relying on one single attribute alone. To investigate this possibility further, the next chapter will explore the development of a clinical formula that uses a number of individual tumour characteristics to provide the clinician with an improved ability to predict patient outcome after surgery.

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Chapter 6:

Development of a nomogram to predict the outcome for patients with soft tissue sarcoma

6.1 Introduction

In the previous chapters, variations in vascular endothelial growth factor (VEGF) and decorin were found to be associated with both recurrence of the tumour and with patient survival after surgery. These results suggest that evaluation of these proteins could be used to help predict patient outcome after surgical resection of a soft tissue sarcoma (STS). The ability to predict whether an individual STS has a higher or lower potential to recur after surgery would greatly assist patient management. This ability could allow a clinician to decide whether an individual patient is likely to benefit from adjuvant chemotherapy or radiotherapy, or whether additional surgery should be performed to remove residual tumour from the wound bed. Having confidence that an individual patient is safe from recurrence would also provide considerable reassurance to pet owners.

Various methods have been developed over the years to help clinicians predict the prognosis for a patient with cancer.[1] Historically, the gold standard for prognostication in human oncology is considered to be the tumour, node, metastasis (TNM) system.[2, 3] This system has been

described for most forms of human cancer since 1953,[3] and was first applied to veterinary oncology in 1980.[4] The TNM system allows categorisation of patients with a different extent of local and distant disease, and offers an ability to stratify the outcome for a patient based on the presenting characteristics of their tumour. Despite the widespread acceptance of the TNM system by the oncology community as an effective method to distinguish patients with different burdens of disease, there are several limitations to the TNM system.[2] This is, in part, because it is founded on the basis that prognosis is directly related to overall tumour burden and an orderly anatomical progression of disease.[5] It is now increasingly understood that many elements of individual tumour biology such as mitotic rate, genetic and histologic characteristics play an increasingly important role in overall prognosis,[1] and these are not always accounted for in the TNM system.

A TNM system has been described for canine STS, but this has not been validated in a clinical setting.[6] Even without this validity, there are notable deficiencies to the classification system that limit its reliability as a predictive tool. For example, within the "T" (tumour) profile, tumour size is the only characteristic used to distinguish different patient subsets. While size does play an influencing role in the prognosis of canine STS,[7, 8] there are several other criteria that are also known to be influential on recurrence including tumour grade, mitotic index, and the percentage of tumour necrosis.[9, 10] Another major limitation of the existing TNM system for canine STS is that it presumes prognosis is determined by the presence of nodal or distant metastasis, which is at odds with the clinical reality of this disease. While

metastatic spread will occur in a proportion of dogs with STS, it is recognised that the majority of dogs that die from STS will be euthanased because of the local impact of their disease rather than the development of metastasis.[11]

In Chapter 4, combining the results of both VEGF and decorin immunostaining classifications appeared to improve the ability to identify the subsets of tumours with either highly favourable or highly unfavourable outcomes. This lends support for a strategy where a combination of different tumour characteristics or prognostic markers could help predict individual tumour behaviour. This conclusion is consistent with developments occurring in the management of human cancer, where a variety of different techniques are being explored to try and improve the accuracy of prognostic predication.[12, 13] Amongst these techniques has been the re-emergence of the nomogram.[14, 15]

A nomogram (also called a nomograph, alignment chart or abaque) is a calculating device that allows the approximate graphical computation of a mathematical function.[5] The field of nomography was invented in 1884 by the French engineer Philbert Maurice d'Ocagne. In an age before pocket calculators and computers, the nomogram became a vital tool for many industries because it allowed quick and accurate computations of complex formulae. The pictorial element of the nomogram allowed a user to derive a reliable solution without having to understand the complex mathematical formulae behind the interface. For more than 75 years, the nomogram was a vital tool for a variety of industries, in particular the railways, astronomy, aeronautics and the military. In more recent years, the nomogram has re-

emerged within the medical field as a potential tool to help patients and doctors utilise complex statistical equations to derive an accurate individual risk assessment for patients with a variety of conditions, including cancer.[14]

The owner of a dog with cancer typically wants to know what their prognosis will be as a result of treatment. Traditionally, a clinician will use their knowledge of the oncology literature, combined with their own experiences, to help determine the potential prospects for an individual patient with cancer. There are a virtually endless supply of clinical studies that have sought to identify different features that help differentiate a cancer patient with a "good prognosis" from a "bad prognosis". The statistical methods used in these papers utilise various forms of logistic regression equations to determine the probability that a certain tumour characteristic, or combination of characteristics, will influence a particular outcome, for example death or tumour recurrence, for an individual patient. These probabilities are published as odds or hazard ratios (HR), with a figure greater than 1.0 indicating there is a higher likelihood for that outcome to occur in a patient with a given tumour characteristic. Conversely, an HR less than 1.0 indicates a lower likelihood for that outcome to occur. An experienced veterinarian familiar with the literature will know that the HR for recurrence of a STS larger than 5cm is 1.8, almost doubling the risk for recurrence when compared to a 1cm tumour.[1] They will also be aware that a grade 3 STS has a HR of 5.8 for recurrence compared to a grade 1 STS. However, HRs have been published for a range of tumour characteristics, including the results of histologic, immunohistochemical or molecular

analysis of the tumour. Because an individual patient with cancer will present with a unique combination of tumour characteristics it is challenging, if not impossible, for a clinician to perform the complicated multi-parameter logistical regression calculations necessary to assimilate the combined influence of each independent tumour variable into an expected outcome. Against this clinical background, the nomogram has emerged as a potential tool to allow a clinician to utilise complex statistical equations to predict a binary outcome from a combination of risk factors.[14]

In human oncology, nomograms have been developed for a variety of tumour types and clinical situations. For example, nomograms have been developed to estimate recurrence,[16] survival outcomes,[17, 18] the benefit of adjuvant therapy[19, 20] and also the impact of a particular treatment on quality of life.[21, 22] Nomograms have been developed to determine the risk for a patient having an incomplete resection if a conservative surgical strategy is employed,[23] or for neoplastic cells to be present within draining lymph nodes.[24] Nomograms have also been developed to better identify patients who may benefit from more extensive surgery.[25] In one study, a predictive nomogram was shown to be significantly more reliable at determining the risk of cancer progression for an individual patient than the clinical judgement of the specialist clinician alone.[26]

To date, nomograms have not been utilised in veterinary medicine to support clinical decisions. Therefore, the aim of the present study was to determine if a nomogram could be used to predict whether an individual STS was likely to recur after surgical resection. After development of the nomogram, the

predictive accuracy was compared with the actual outcome for the patient. This was also compared with the accuracy of predicting outcome using the HR for individual tumour characteristics alone. The hypothesis was that the nomogram would be more accurate at determining patient outcome than the prognosis that could predicted from individual tumour characteristics.

6.2 Materials and Method

6.2.1 Patient data

Separate datasets were used to develop the two nomograms created in this current study. The first dataset was derived from the population of 350 soft tissue sarcoma established in Chapter 3. This dataset was called "Clinical". Because accurate nomogram construction requires no missing or incomplete information for the variables used in the analysis, any cases with unknown or missing values were excluded. The final Clinical dataset therefore included 170 cases. The second dataset utilised the clinical cases that were used to determine the immunostaining characteristics for VEGF and decorin as detailed in Chapter 4. This dataset was termed "IHC". Once again, any cases with unknown or missing values were excluded. The final IHC dataset therefore included 82 cases.

To allow development of well-calibrated and validated nomograms, each model is ideally built using a training cohort of data, and then validated against an independent validation cohort. To establish these two required cohorts, the CRAN package "sampling" in R (R version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria) was used to randomly select 68

cases from the Clinical dataset. These selected cases were used to create the validation cohort, called “Clinical_validate”. The cases remaining now created the larger training cohort which consisted of 102 cases; this dataset was renamed as “Clinical_train”.

Because of the small number of cases in the IHC database, it proved impossible to separate the dataset into two and still retain a meaningful number of events within each cohort. For this reason, it was not possible to create an independent cohort for the IHC nomogram to permit external validation.

6.2.2 Patient demographics and risk analysis of individual variables

All statistical analyses were performed with SPSS (Version 25, IBM Statistics, USA). Local recurrence of the tumour within 3 years was the defined end point for the study. The disease-free interval (DFI) was defined as the time from surgery to the time when recurrence was identified by the referring vet. Patients were censored if they had died prior to the end point of the study and no tumour recurrence had been noted at that time, based on clinical records of the referring veterinarian.

The Kaplan Meier method was used to compare DFI according to age, palpable characteristics, tumour size, histological characteristics (i.e. differentiation, necrosis, mitotic score, grade), and the development of local tumour recurrence. Finally, Cox regression analysis was performed to identify categories of significance, and their hazard ratios, for patients whose

tumours recurred within 3 years of surgery. A value of $p<0.05$ was considered significant.

6.2.3 Using a ROC curve to evaluate the predictive accuracy of individual tumour characteristics

For each category showing significance with Cox regression analysis, the test result was plotted against actual tumour recurrence in a receiver-operating-characteristic (ROC) curve. The ROC curve provides an ability to determine the ability of a diagnostic test to discriminate between affected and non-affected patients.[27] When the outcome is binary (such as tumour recurrence), a diagnostic test may reliably predict whether an event actually occurred (true positive (1) or true negative (0)), or wrongly predicts an outcome which does not occur (false positive or false negative). An imperfect test will produce an equal number of false-positive and false-negative results, providing a predictive ability that is no better than chance (i.e. 50:50). If the binary predictions of a diagnostic test are presented graphically, a diagonal line will be generated between the origin (0,0) and the top right quadrant of the graph (1,1). The ROC curve thus provides a visual representation of the clinical utility of a particular test. Calculation of the area-under-the-curve (AUC) of the ROC curve line also provides an objective measure of reliability of the diagnostic test.[27] A perfect test will have an AUC of 1.0 whereas the imperfect test will have an AUC of 0.5. The aim for a diagnostic test is to have an AUC as close as possible to 1.0.

Using co-ordinates from the ROC curve, a cut-off value for 3 year local recurrence probability was determined by calculating the differential positive

rate using the following formula: [sensitivity – (1-specificity)].[27] This allowed determination of a probability value that provided an optimal balance of sensitivity and specificity. This enabled a binary recurrence outcome (i.e. yes or no) to be predicted, based on the actual test result. By comparing this predicted outcome with the actual outcome in a 2x2 table, it was possible to calculate Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Values for both the "Clinical_train" and the "IHC" nomograms.

6.2.4 Nomogram construction

To identify the independent predicators of time-to-event outcome that should be used in nomogram construction, multivariable Cox regression analysis was performed on all recorded clinical variables in the "Clinical_train" dataset, including age, size of tumour, palpable characteristics, location, as well as histological characteristics of the tumour including grade, differentiation, necrosis, mitoses and mitotic rate. Backward selection of variables was performed to obtain the model with the best fit. Due to the small size of the dataset, variables were selected for use in the model if their p value was <0.15.

Following selection of the independent variables to be used in the model, nomograms were constructed using the 'rms' and 'survival' packages available in R (R version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria), as described by Harrell.[28] The code for nomogram construction is shown in Table 6.1.

These above steps were then repeated using the "IHC" dataset. The variables used for development of the multivariable logistic equation included age, size of tumour, palpable characteristics, location, the histological characteristics of the tumour (i.e. grade, differentiation, necrosis, mitoses and mitotic rate), as well as the immunostaining scores for VEGF and decorin.

Table 6.1

Example of the R code used to develop the clinical nomogram. This utilised the 'rms' and 'survival' packages in R.

```
#RMS load data and nomogram construction
nomo <- read.csv("Clinical_train_random.csv", TRUE)
library(rms)
mod.Cox <- cph(Surv(DFI, CENSR) ~Palpnew + Mitoticrate + Necrosis,
nomo,surv=TRUE)
ddist <- datadist(nomo)
options(datadist='ddist')
surv.Cox <- Survival(mod.Cox)
nom.Cox <-
nomogram(mod.Cox,fun=list(function(x)surv.Cox(1095,x)),funlabel=c(
"3-year DFS"),lp=FALSE)
plot(nom.Cox)

#RMS validation of Cox model using validation dataset
nomo_valid <- read.csv("Clinical_train.csv", TRUE)
fit_valid <- cph(Surv(DFI,CENSR) ~Palpnew + Mitoticrate +
Necrosis, nomo_valid,x=TRUE, y=TRUE)
validate(fit_valid, method="boot", B=40, bw=FALSE,
rule="aic",type="residual", sls=.05, aics=0, force=NULL,
estimates=TRUE, pr=FALSE, dxy=TRUE, u, tol=1e-9)

#RMS nomogram of IHC training data
nomo <- read.csv("IHC_test all.csv", TRUE)
library(rms)
mod.Cox <- cph(Surv(DFI, CENSR) ~VEGF + Decorin + Mitoticrate +
Age, nomo,surv=TRUE)
ddist <- datadist(nomo)
options(datadist='ddist')
surv.Cox <- Survival(mod.Cox)
nom.Cox <-
nomogram(mod.Cox,fun=list(function(x)surv.Cox(1095,x)),funlabel=c(
"3-year DFS"),lp=FALSE)
plot(nom.Cox)

#RMS validation of Cox model using IHC_validation dataset
nomo_valid <- read.csv("IHC_test all.csv", TRUE)
fit_valid <- cph(Surv(DFI, CENSR) ~VEGF + Decorin + Mitoticrate +
Age, nomo_valid,x=TRUE, y=TRUE)
validate(fit_valid, method="boot", B=40, bw=FALSE, rule="aic",
type="residual", sls=.05, aics=0, force=NULL, estimates=TRUE,
pr=FALSE, dxy=TRUE, u, tol=1e-9)
```

6.2.5 Statistical validation of the nomograms

The performance of both the "Clinical_train" and the "IHC" nomogram was assessed by determining the concordance index (*C*-index). The *C*-index is a measure of goodness of fit for binary outcomes in a logistic regression model and gives the probability for whether the predicted outcome agrees with the observed outcome. The difference between these two measures is Somer's D (Dxy) value. The *C*-index was calculated from Dxy using the following formula: *C*-index = *0.5 * (Dxy + 1)*.

With nomogram development, it is common practice to use resampling methods to enable validation of the predictive performance of the Cox model used in the nomogram. For this study, the Bootstrap method was employed, with the model iteratively applied to 200 randomly created datasets using cases selected from the original cohort.[29] The results generated by the 'rms' *validate* function in 'R' compares the predictive ability of the original data with the mean of those derived by bootstrapping. The difference between the original *C*-index and the average derived by bootstrapping is an estimate of the overfit, or optimism.

6.2.6 Validation of the nomograms using an independent dataset

The performance of the nomogram was next assessed by generating the *C*-index using the independent dataset "Clinical_valid". The bootstrap method was again employed, with the model iteratively applied to 200 randomly selected samples from the independent cohort. The *C*-index was calculated from Dxy, using the formula as above.

6.2.7 Nomogram validation by manual calculation of values

Following creation of the nomogram, the probability of outcome was manually calculated for each case in the original "Clinical" and "IHC" datasets. Previously excluded cases from the original population of 350 soft tissue sarcoma established in Chapter 3 were included if their "unknown" variable was not required in the nomogram calculation. For the Clinical dataset, this enabled the addition of another 62 cases where 'size' had been classified as unknown; the final cohort available for manual validation of the Clinical nomogram was now 232 cases. No additional cases were included in the IHC dataset for manual validation of the IHC nomogram.

6.2.8 Sensitivity, Specificity and ROC validation of the nomograms

The probability score for predicted tumour recurrence derived from the nomogram was then plotted against actual tumour recurrence in a Receiver Operating Characteristic (ROC) curve.

Using co-ordinates from the ROC curve, a cut-off value for 3-yr local recurrence probability was determined. This cut-off value was then applied to the local recurrence probability that had been determined for all patients in both the "Clinical" and the "IHC" datasets. This enabled a binary recurrence outcome to be predicted. By comparing this predicted outcome with the actual outcome in a 2x2 table, Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Values could be calculated for both the "Clinical" and the "IHC" nomograms. The area under the curve (AUC) of the

ROC curve line was also calculated and compared with the *C*-index generated by the statistical method described above.

6.3 Results

6.3.1 Clinical train dataset

6.3.1.1 Patient Demographics:

The "Clinical_train" dataset contained a total of 102 patients. During the study period, tumour recurrence occurred in 27 patients (27%), with a median DFI of 557 days (range 28 – 1068 days). From Kaplan Meier analysis, the palpable characteristics of the tumour (fixed vs. mobile) and various histological characteristics (necrosis, mitotic rate and grade) were all found to have a significant influence on recurrence.

Calculated hazard ratios for each individual clinical parameter was determined by univariate Cox regression analysis. These results suggested that a fixed tumour was 4.4 times more likely to recur than a discrete, mobile tumour; a high-grade tumour was 2.6 times more likely to recur than a low-grade tumour; and a tumour with a mitotic index of 3 was 1.9 times more likely to recur than a tumour with a mitotic index of 1 (Table 6.2).

Based on the ROC curves generated for each clinical parameter, the predictive ability to determine the actual outcome for patients was considered to be poor for tumour size, differentiation, mitotic rate, necrosis and age; the AUC for these variables was calculated to be between 0.49 and 0.60. Only the variables "Palpable characteristics" and "Grade" showed some

ability to distinguish patients, with an AUC of 0.68 and 0.67 respectively (Table 6.3).

Table 6.2:
Demographics – Clinical_train dataset

	Mean	Median	Signif.	HR (95% CI)
Disease free interval (days)	764.57	655		
Recurrence Recur = 27 (27%) No recur = 75 (73%)				
Differentiation 1 = 54 (53%) 2 = 38 (37%) 3 = 10 (10%)			p= 0.7	1.2 (0.7 - 2.2)
Mitotic rate **1 = 80 (78%)** **2 = 14 (14%)** **3 = 8 (8%)**			P = 0.03	1.9 (1.1 - 3.2)
Necrosis 0 = 74 (73%) 1 = 23 (23%) 2 = 5 (5%)			P = <0.001	2.8 (1.6 – 5.0)
Grade 1 = 70 (69%) 2 = 24 (24%) 3 = 8 (8%)			P = <0.001	2.6 (1.6 - 4.3)
Age 3 – 16 years	9.657	9.5		1.0 (0.9 - 1.2)
Size < 1cm = 6 (6%) 1-5cm = 57 (56%) 5cm = 39 (38%)			P = 0.1	1.0 (0.1 - 6.5)
Palpable Mobile = 49 (48%) Fixed = 53 (52%)			P = 0.001	4.4 (1.8 - 11.05)

Table 6.3:
AUC of ROC curve for individual parameters in the Clinical_train dataset

Parameter	AUC	significance	95% confidence interval
Size	0.581	p = 0.215	0.45 - 0.711
Palpable characteristics	0.676	p = 0.007	0.561 - 0.79
Differentiation	0.534	p = 0.606	0.406 - 0.661
Mitotic rate	0.584	p = 0.197	0.453 - 0.715
Necrosis	0.604	p = 0.109	0.472 - 0.737
Grade	0.666	p = 0.011	0.541 - 0.792
Age	0.488	p = 0.856	0.376 - 0.6
Mitoses	0.629	p = 0.047	0.501 - 0.757

Using coordinates from the ROC curves, the cut-off values for "palpable characteristics" and "grade" was determined to be "fixed, immobile" and "grade 2 or grade 3" tumours respectively. When this predicted outcome was compared to the actual outcome, the following results were obtained:

Palpable characteristics: A true positive result was obtained in 21 patients, but a further 32 patients were wrongly predicted to experience recurrence when they did not (i.e. false positive). Accurate prediction of no recurrence was made in 43 patients (i.e. true negative), but tumours recurred in 6 patients when the test results suggested it would not (i.e. false negative). Overall, this gave a sensitivity of 78%, a specificity of 57%, a positive predictive value of 40%, and a negative predictive value of 88%

Grade: A true positive result was obtained in 15 patients, but a further 17 patients were wrongly predicted to experience recurrence when they did not. Accurate prediction of no recurrence was made in 58 patients, but tumours recurred in 12 patients when the test results suggested it would not. Overall,

this gave a sensitivity of 56%, a specificity of 77%, a positive predictive value of 47%, and a negative predictive value of 83%.

6.3.1.2 Nomogram construction: Clinical

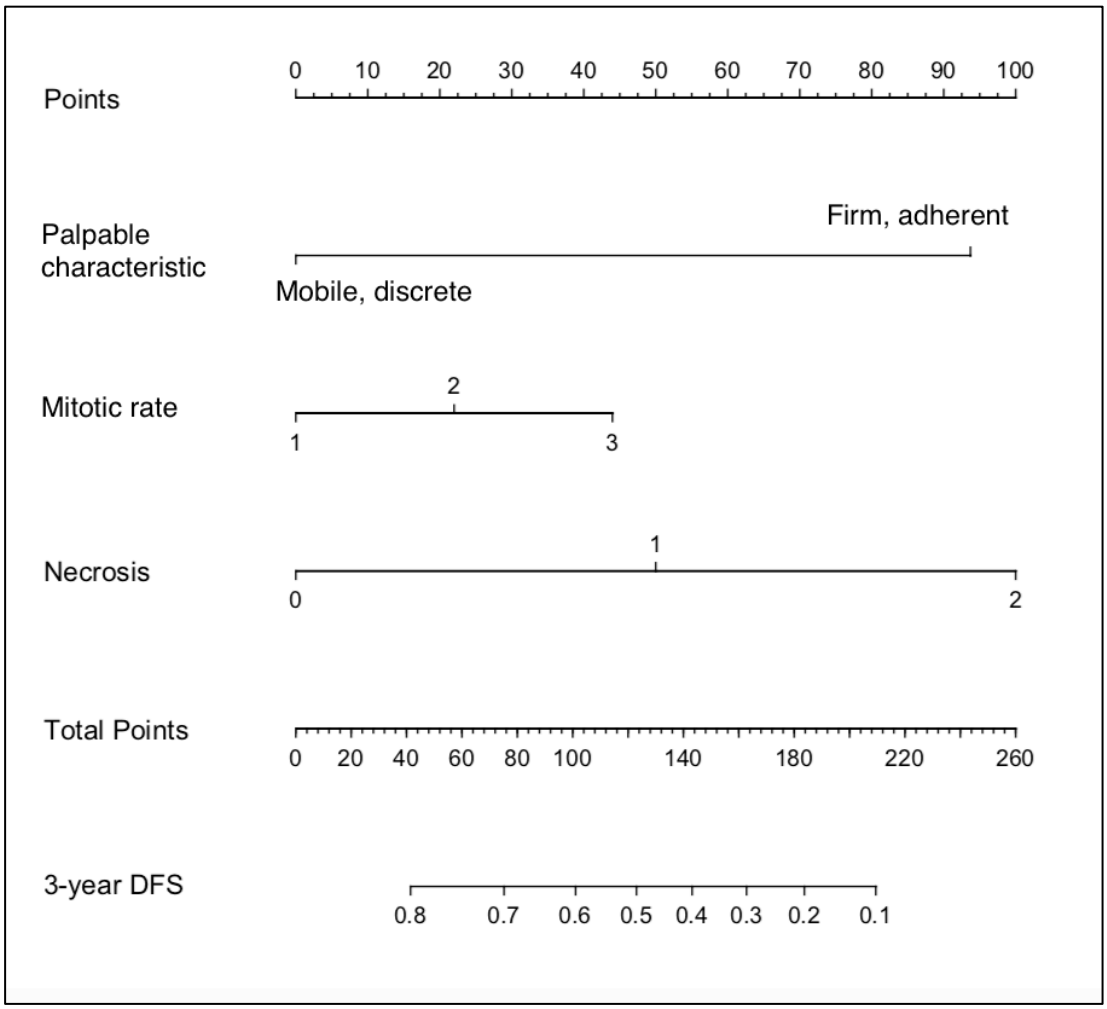
Using backward selection multi-variable Cox regression analysis, the optimal variables for use in the nomogram was determined, as shown in Table 6.4.

Table 6.4
Multivariable COX regression analysis on Clinical_train database to identify characteristics to be used in the nomogram

	Clinical characteristic	**Significance** (p value)	**HR**	**95.0% CI for HR** Lower	Upper
1	Well-differentiated Moderately differentiated Poorly differentiated	0.995 0.949 0.966	- 0.967 1.054	- 0.346 0.096	- 2.7 11.595
2	Size (<1cm) Size (1-5cm) Size (>5cm)	0.778 0.329 0.611	- 1.623 1.334	- 0.613 0.44	- 4.296 4.048
3	Age	0.79	0.98	0.846	1.136
4	Mitoses	0.731	1.011	0.95	1.075
5	Grade 1 Grade 2 Grade 3	0.657 0.736 0.389	- 0.783 0.303	- 0.188 0.02	- 3.253 4.598
6	Palpable (discrete) Palpable (firm, immobile Mitotic rate score 1 Mitotic rate score 2 Mitotic rate score 3 Necrosis score 1 Necrosis score 2 Necrosis score 3	 0.035 0.015 0.11 0.007 0.181 0.156 0.318	 2.403 - 2.141 5.08 - 0.49 2.128	 1.065 - 0.841 1.571 - 0.183 0.483	 5.421 - 5.446 16.422 - 1.313 9.377

Based on these results, “Palpable characteristic”, “Mitotic Rate” and “Necrosis” were used to generate a nomogram to calculate the probability for being tumour free at 3 years (Figure 6.1).

Figure 6.1:
Nomogram developed from the Clinical_train dataset



6.3.1.3 Statistical validation of the clinical nomogram

Validation of the Cox model using the training dataset (Clinical_train) generated a Dxy value of 0.45, which equated to a *C*-index of 73%. With bootstrapping, the Dxy value was 0.44, which equated to a *C*-index of 72%.

From these values, the optimism-corrected estimate of Dxy was 0.4, giving a *C*-index of 70%.

When validation of the Cox model was performed using the independent dataset (Clinical_valid), the Dxy value was 0.23, which equated to a *C*-index of 61%. With bootstrapping, the Dxy value was 0.14, which equated to a *C*-index of 57%. From these values, the optimism-corrected estimate of Dxy was 0.03, equating to a *C*-index of 51%.

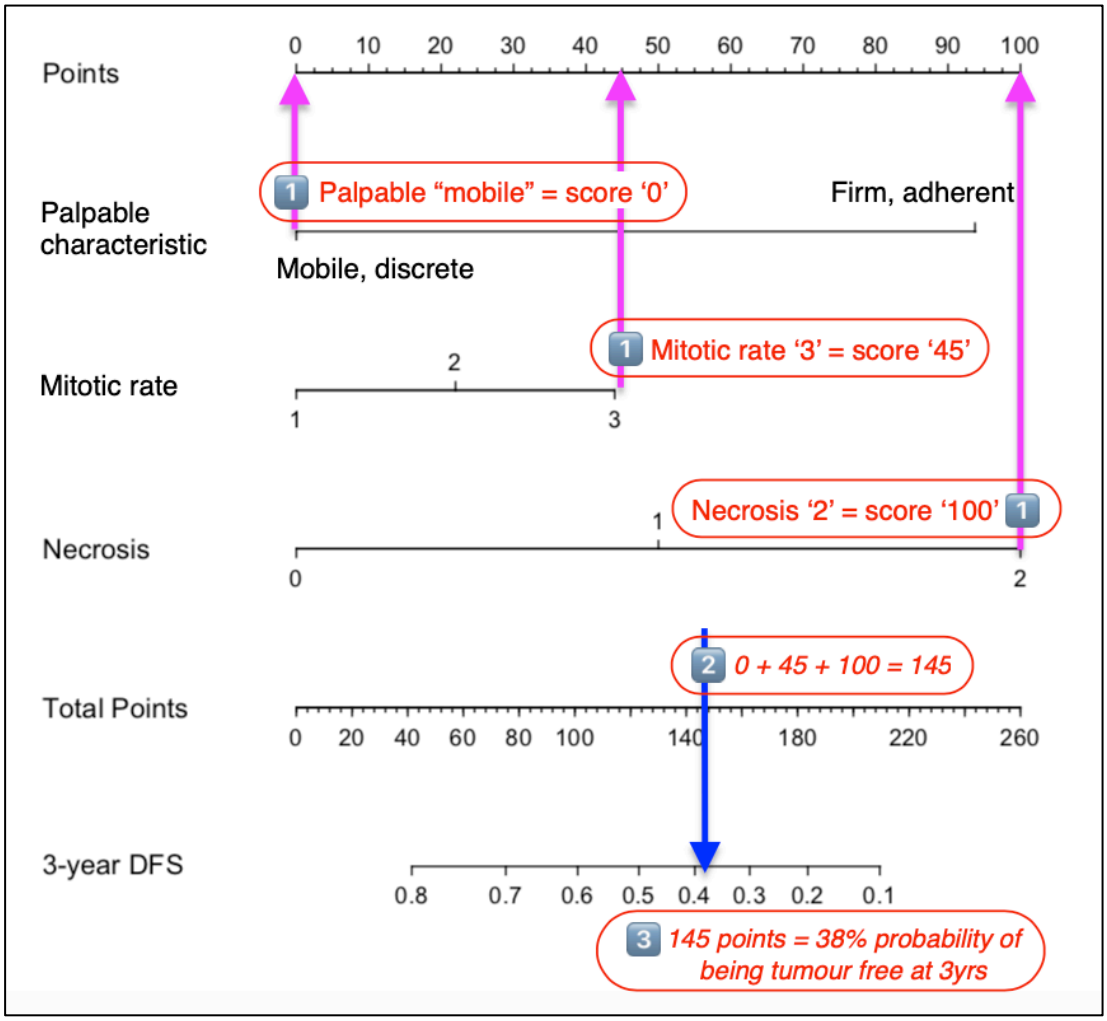
6.3.1.4 Manual validation of the clinical nomogram

Use of the nomogram is relatively simple and involves 3 separate steps, as shown in Figure 6.2. Firstly, using the scale for each variable, the 'Points' scale at the top of the chart is used to determine the individual value for each patient. Next, the 'total score' of all variables are totalled. Finally, the 'Total points' scale is used to determine the 'probability of outcome', with values read from the 3-year DFS (disease free survival) probability scale.

Using the probability values generated from the nomogram for each case in the "Clinical" database, the resulting ROC curve gave an AUC of 0.67 (95% CI 0.6 – 0.75, p= <0.0001) (Figure 6.3).

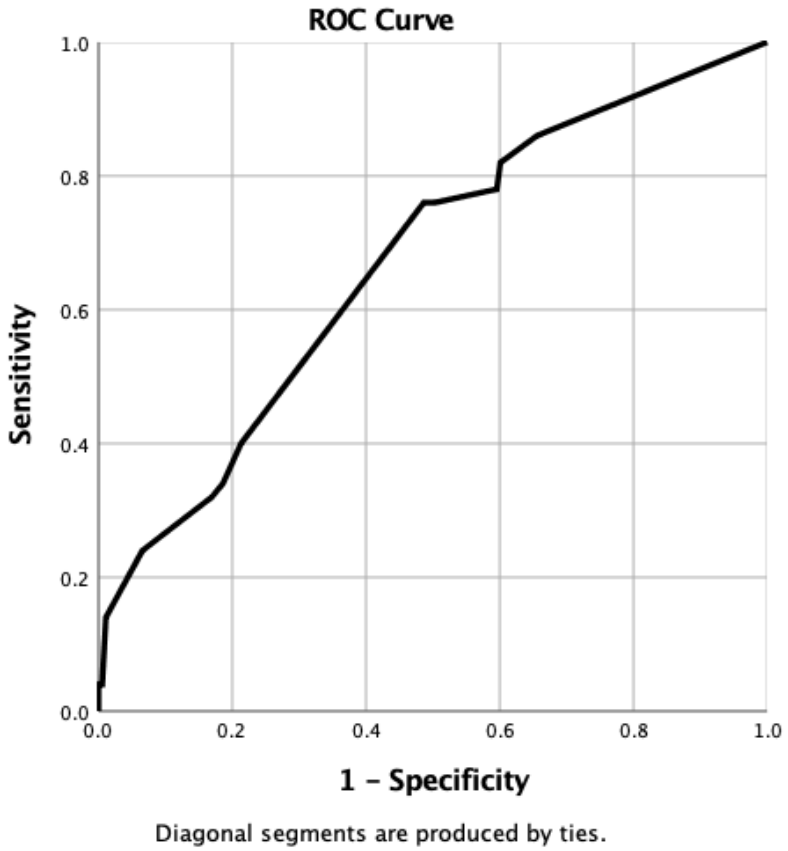
Using coordinates of the ROC curve, the optimal cut-off value of probability to provide a binary predictor of tumour recurrence within 3 years was determined to be >85%. When this value was applied to all cases in the Clinical dataset, the nomogram was found to have correctly identified 41 patients where recurrence occurred (true positive), but incorrectly predicted

Figure 6.2:
Steps to using a nomogram. 1) determine the POINTS scored for each characteristic defined in the nomogram 2) Total these points and identify this value on the TOTAL POINTS scale. 3) The 3-year disease free interval is then determined using the proportional scale that is in line with the value identified in the previous calculation.



recurrence in 110 patients when no recurrence was observed (false positive). The nomogram accurately predicted tumour-free survival in 73 patients (true negative), but failed to predict recurrence in 9 patients (false negative). Overall, the sensitivity, specificity, positive predictive and negative predictive values for the Clinical nomogram were 82%, 40%, 27% and 89% respectively.

Figure 6.3:
ROC curve generated from probabilities derived from Clinical nomogram



6.3.2 IHC dataset

6.3.2.1 Patient Demographics

The IHC dataset contained a total of 82 patients (Table 6.4). Tumour recurrence developed in 26 patients (32%), with a median DFI of 655 days (range 28 – 1098 days). From Kaplan Meier analysis, immunostaining of VEGF, necrosis and the palpable characteristics for the tumour were all found to be influential on recurrence.

Calculated hazard ratios for each individual clinical parameter, as determined by univariate Cox regression analysis, are shown in Table 6.5. These results

Table 6.5:
Demographics - IHC dataset

	Mean	Median	Range	Signif.	HR (95% CI)
Disease free interval (days)	772.51	655			
Recurrence Recur = 26 (32%) No recur = 56 (68%)					
VEGF score Low = 43 (52%) High = 39 (47%)				**p= <0.001**	8.4 (2.9 - 24.4)
Decorin score 1 = 26 (32%) 2 = 22 (27%) 3 = 34 (42%)				p= 0.7	
Differentiation 1 = 31 (38%) 2 = 40 (49%) 3 = 11 (13%)				p= 0.7	
Mitotic rate 1 = 61 (74%) 2 = 12 (15%) 3 = 9 (11%)				p= 0.6	
Necrosis 0 = 54 (66%) 1 = 23 (28%) 2 = 5 (6%)				**p= 0.003**	- 1.1 (0.4 - 2.7) 7.2 (1.9 - 26.7)
Grade 1 = 46 (56%) 2 = 27 (33%) 3 = 9 (11%)				p= 0.5	
Age	9.77	10	12		
Size < 1cm = 2 (2%) 1-5cm = 45 (55%) >5cm = 21 (26%)				p= 0.2	
Palpable Mobile = 32 (39%) Fixed = 45 (55%)				**p= 0.03**	- 2.7 (1.1 - 6.8)

suggested that a tumour with diffuse immunostaining for VEGF was 8.4x more likely to recur than one with low immunostaining. A tumour with >50% necrosis was 7.2x more likely to recur than one with minimal necrosis, and a fixed tumour was 2.7x more likely to recur than a mobile one.

Using the ROC curve, the predictive ability of individual test characteristics to reliably determine the actual outcome for patients was considered to be poor. For the variables decorin, differentiation, mitotic rate, necrosis, grade, age and tumour size, the AUC was calculated to be between 0.49 and 0.64 (Table 6.5). Only VEGF showed some ability to distinguish patients, with an AUC of 0.79 (Figure 6.4).

Using coordinates from the ROC curves, the cut-off value for VEGF to determine a binary decision for recurrence was "1". When this predicted outcome was compared to the actual outcome, true positive results were obtained in 22 (27%) patients, but a further 17 (21%) patients were wrongly predicted to experience recurrence when they did not (false positive). Accurate prediction of no recurrence was made in 39 (48%) patients (true negative), but tumours recurred in 4 (5%) patients when the test results suggested it would not (false negative). Overall, this gave a sensitivity of 84%, a specificity of 70%, a positive predictive value of 56%, and a negative predictive value of 90%.

Figure 6.4:
ROC curve generated from diagnostic reliability for VEGF on predicting tumour recurrence.

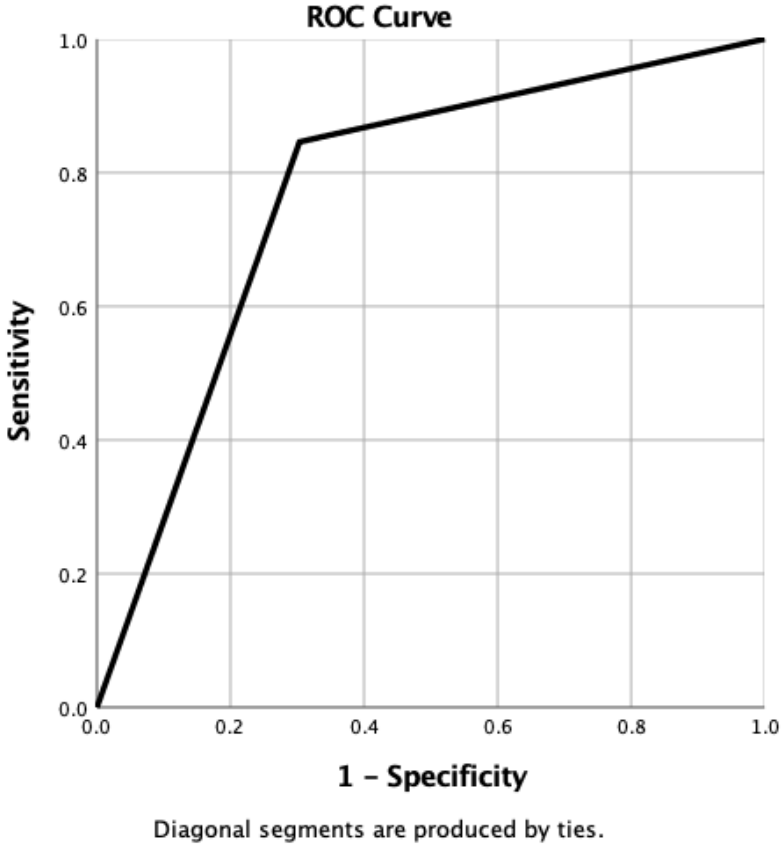


Table 6.5:
AUC of ROC curve for individual parameters in the IHC dataset

Parameter	AUC	significance	95% confidence interval
VEGF	0.786	p = <0.001	0.677 - 0.895
Decorin	0.534	p = 0.628	0.398 - 0.669
Differentiation	0.488	p = 0.863	0.354 - 0.622
Mitotic rate	0.517	p = 0.804	0.379 - 0.655
Necrosis	0.54	p = 0.572	0.399 - 0.68
Grade	0.506	p = 0.936	0.37 - 0.642
Age	0.626	p = 0.072	0.5 - 0.752
Size	0.52	p = 0.779	0.374 - 0.665

6.3.2.2 Nomogram construction: IHC

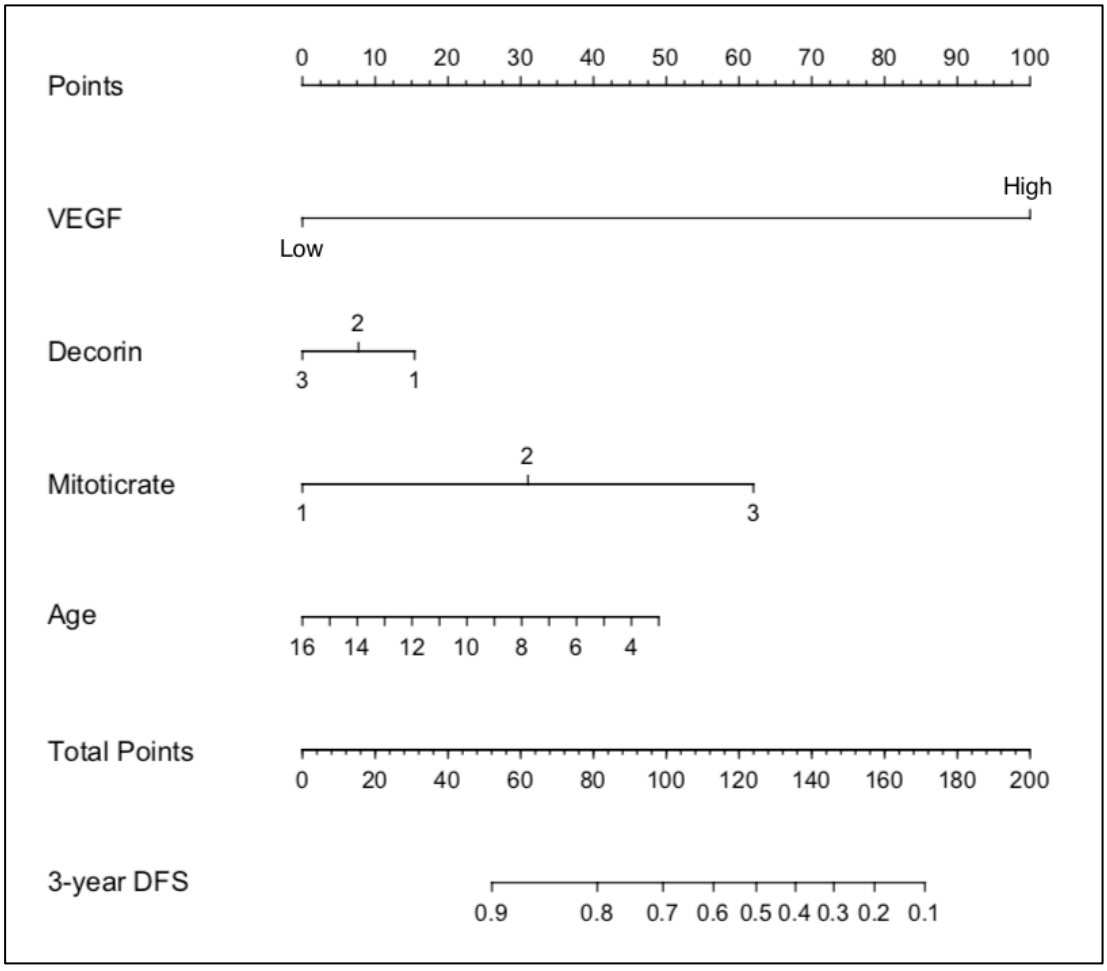
The stepwise determination of optimal variables using backward selection multi-variable Cox analysis is shown in Table 6.6. Based on these results, four variables - VEGF, decorin and mitotic rate and age - were used to generate a nomogram to calculate the probability for being tumour free at 3 years (Figure 6.5).

Table 6.6:
Stepwise backward selection of variables in the IHC database using Cox regression analysis eventually identified four characteristics of appropriate significance to be used in the nomogram

	Clinical characteristic	Significance (P value)	HR	95.0% CI for HR	
				Lower	Upper
1	Well-differentiated Moderately differentiated Poorly differentiated	0.9 0.7 0.9	- 1.3 0.6	- 0.314 0.004	6
2	Size (<1cm) Size (1-5cm) Size (>5cm)	0.8 0.3 0.6	- 1.623 1.334	- 0.613 0.44	- 4.296 4.048
3	Grade 1 Grade 2 Grade 3	0.6 0.7 0.3	- 0.723 0.231	- 0.135 0.014	- 3.882 3.786
4	Necrosis score 1 Necrosis score 2 Necrosis score 3	0.3 0.3 0.4	- 0.564 1.842	- 0.198 0.437	- 1.607 7.764
5	Palpable (discrete) Palpable (firm, immobile)	0.2	- 1.769	- 0.675	- 4.635
6	VEGF low VEGF high Decorin type 1 Decorin type 2 Decorin type 3 Mitotic rate score 1 Mitotic rate score 2 Mitotic rate score 3 Age	<0.0001 0.1 0.9 0.1 0.01 0.6 0.002 0.1	- 31.25 - 1.097 0.397 - 0.727 25.271 0.856	- 5.197 - 0.394 0.134 - 0.207 3.257 0.71	- 187.903 - 3.06 1.18 - 2.551 196.062 1.031

Validation of the Cox model using all of the cases in the IHC dataset generated a Dxy value of 0.6. This equated to a *C*-index of 80%. With bootstrapping, the D value was 0.61, which equated to a *C*-index of 81%. This provided an optimism-corrected *C*-index of 76%.

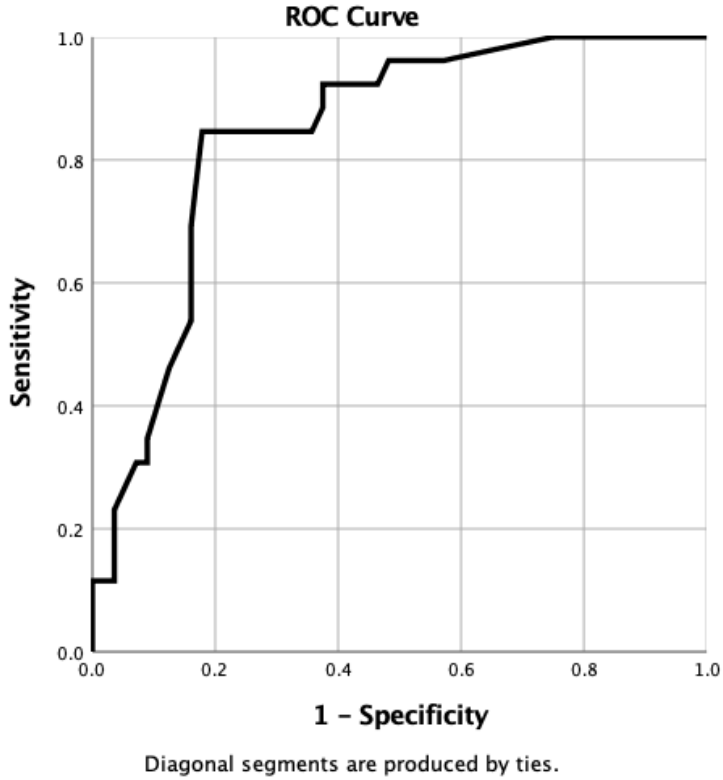
Figure 6.5:
Nomogram developed from the IHC dataset



6.3.2.3 Manual validation of the IHC nomogram

Using the probability values generated from the nomogram for each case in the IHC database, the resulting ROC curve gave an AUC of 0.84 (95% CI 0.76 – 0.93, p= <0.0001) (Figure 6.6).

Figure 6.6:
ROC curve generated from probabilities derived from IHC nomogram



Using coordinates of this ROC curve, the optimal cut-off value of probability to provide a binary predictor of tumour recurrence within 3 years was determined to be >90%. When this value was applied to all cases in the IHC dataset, the nomogram was found to have correctly identified 25 patients where recurrence occurred (true positive), but incorrectly predicted recurrence in 31 patients when no recurrence was observed (false positive). The nomogram accurately predicted tumour-free survival in 25 patients (true negative) but failed to predict recurrence in 1 patient (false negative). Overall, the sensitivity, specificity, positive predictive and negative predictive values for the IHC nomogram were 96%, 45%, 45% and 96% respectively.

6.3.3 Summary of results

When the predictive abilities of individual tumour characteristics are compared with the results of both the clinical and IHC nomogram, the IHC nomogram shows clear superiority in providing a reliable prediction of outcome with an AUC of 0.84 (Table 6.7).

Table 6.7:
Comparison of predictive abilities of individual tumour characteristics are compared with the results of both the clinical and IHC nomogram

	C-index	AUC of ROC curve (95% CI)	Sens	Spec	PPV	NPV
Individual Characteristics						
Palpable Grade only VEGF		0.68 0.67 0.79	78% 56% 84%	57% 77% 70%	40% 47% 56%	88% 83% 90%
Nomograms						
Clinical nomogram *Training dataset* *Validation dataset*	71% 51%	0.67 (0.6 – 0.75)	82%	40%	27%	89%
IHC nomogram	75%	0.84 (0.76 – 0.93)	96%	45%	45%	96%

Key: C-index = concordance index
AUC of ROC curve = area under curve of receiver operating curve
Sens = Sensitivity
Spec = Specificity
PPV = Postive predictive value
NPV = Negative predictive value

6.4 Discussion

The results from this study suggest that a nomogram may be useful to help predict the likelihood for a STS to recur after surgery in a dog. Of the two nomograms developed in the current study, inclusion of the

immunohistochemical staining characteristics developed in Chapter 4 significantly improved the reliability of the prediction provided by the model. While the use of various clinical and histological characteristics of the tumour have been used for many years to help predict potential tumour behaviour, this is the first time the use of a graphical calculating tool such as a nomogram has been described in veterinary medicine.

The purpose of the nomograms developed in this study was to identify dogs whose tumours were more likely to recur after surgery. This endpoint was selected as it is known that local recurrence of the tumour is the most common cause of tumour-related death.[1] If these dogs could be identified earlier, it is possible that their lives could be saved or prolonged by performing a wider resection of the tumour scar, or by providing other adjuvant therapies such as chemotherapy or radiotherapy to prevent progression of their tumour.

An important attribute of any diagnostic test is its ability to provide an accurate prediction of the true disease status of an individual patient. A diagnostic test needs to have an optimal balance between sensitivity and specificity; this will ensure the animals that the test is intended to identify are not overlooked (false-negatives), while animals who do not have the attribute are not inadvertently included (false-positives). From the current study, the AUC of the ROC curve for individual tumour characteristics such as size, age, mitotic rate and necrosis was between 0.5 and 0.6, which suggests their ability to predict which individual was likely to have an undesirable outcome was not much better than flipping a coin. Only the grade and palpable

characteristics of the tumour provided some improved differentiation, but a high degree of uncertainty remained in the prediction. Using these criteria alone, it would be challenging for a clinician to recommend that a dog undergo further treatment when there is up to a 50% chance that the dog has been falsely identified as being 'at-risk' and recurrence will actually never occur.

When several characteristics of the tumour, including palpable characteristics, mitotic rate and necrosis score, were combined into a nomogram using statistical modelling, the ability to predict outcome improved with a sensitivity of 82%, However, because specificity remained poor, there were almost three dogs wrongly suspected of being at risk of recurrence for every dog correctly identified.

It was only when the immunohistochemical characteristics of the tumour were included in the model that the predictive abilities of the nomogram began to demonstrate some degree of clinical utility. However, even in this instance, there was still an almost 40% false positive rate. This would again create challenges for a clinician who needs to decide whether to recommend additional treatment for an individual patient.

Although the nomograms developed in this current study may not, in their existing form, provide a clinician with the precision required to accurately identify patients where recurrence was more likely, the high sensitivity of the IHC nomogram does allow a clinician to accurately identify patients where recurrence is unlikely to occur. Using the IHC dataset, the nomogram accurately predicted tumour-free survival in more than 96% of patients.

Within the study population, the risk of recurrence was almost 30%, and there was no other ability to distinguish the patients where recurrence was likely or unlikely to occur. However, by using the information from the IHC nomogram, a clinician could confidently identify the patients where tumour recurrence would not occur. For the owners of these dogs, progressing from a 30% possibility that recurrence could develop after surgery to an almost 100% certainty that their dog's tumour was not going to recur can provide a tremendous degree of relief.

Nevertheless, the inability of the nomograms to reliably predict which STS will recur is a major weakness, and suggests they lack some vital distinguishing characteristic that would improve differentiation. One of the obvious deficiencies in the data used to develop the nomograms in the current study is the absence of information on the completeness of tumour resection, or the histological margin. It is generally accepted that demonstration of a resection margin that is clear of tumour cells is considered the best predictor for improved local tumour control,[1, 7, 30-36] It is therefore likely that if information on the histological completeness of the surgical margin were included in the nomogram, this would improve the specificity of the nomograms developed in this study.

Despite this lack of information on the surgical margin, it is interesting that the nomograms developed in this study were able to provide a reasonably accurate prediction of tumour recurrence. This lends support to the hypothesis that the status of the surgical margin is not always a definitive guide to a patient's outcome after surgery, with other aspects of tumour

biology influencing the ability for a tumour to regrow after surgery, irrespective of whether the surgeon has successfully removed all of the neoplastic cells. As discussed in Chapter 2, a STS may recur even when the histologic margins have been determined to be complete, and an incomplete surgical margin does not mean tumour recurrence is inevitable. In dogs, recurrence rates for STS of between 5-22% have been reported when a clean resection has been achieved, and no regrowth may occur in up to 83% of patients when incomplete or close resection margins have been described.[7, 37] Similar findings have been reported for human soft tissue sarcoma.[38]

It is therefore an intriguing prospect that the IHC nomogram developed in this study was able to provide a reasonable prediction of tumour outcome after surgery, even though the model incorporated no knowledge about the surgical margin or whether there was persistence of tumour cells within the wound bed. One of the goals of this PhD was to identify predictive markers for STS behaviour that could assist the surgeon in identifying when a STS could be safely treated with a conservative resection, or when an aggressive resection with adjuvant therapy should be considered. The results of the current study raise the possibility that a nomogram could be used to predict the potential for an individual tumour to recur, even before surgery has been performed. Such a strategy could enable a clinician to determine the appropriate surgical margins required for effective management for that individual tumour. There are currently only a limited number of publications in human oncology that describe the development of a nomogram to enable prediction of surgical margins.[23, 39, 40] In one paper, a nomogram was developed to enable better pre-operative stratification of patients due to

undergo breast conserving surgery (BCS) for early-stage breast cancer.[23] Breast conserving surgery is a highly effective strategy for the treatment of women with localised breast cancer and may be preferred over more radical mastectomy procedures due to the reduced morbidity and cosmetic impact of surgery. However, if conservative surgery is performed on a tumour that proves to be more aggressive than was originally suspected, then the patient will need to undergo additional surgery and adjuvant treatment in an effort to regain effective control of their tumour. This situation is the current reality of surgical planning for many forms of cancer: that is, the information necessary to predict the prognosis and best treatment strategy for a patient only becomes available to a clinician once the surgery has already been performed. Ideally, the planning of cancer surgery should be performed in conjunction with appropriate knowledge about the tumour's innate behaviour. This would enable to surgeon to perform an appropriate dose of surgery – with dose equating to the extent of surgical margins performed about the mass – required to achieve successful control of that individual tumour. Evidence from the IHC nomogram developed in the current study suggests that such a strategy could be viable, but further development validation would be required. In reality, oncologic surgeons would need exceedingly good evidence to be convinced that surgical margins could be deliberately reduced about a STS, as the consequences of an incomplete resection could be detrimental for the affected patient.

There have been numerous studies on the use of nomograms to predict various aspects of the clinical decisions surrounding cancer management,[15-20, 23, 24, 26, 39-42] but the actual uptake of these in the clinical setting is

unknown. Each of the studies described above was performed retrospectively, using data from patients who had already undergone surgery. To date, there is only one publication that reports a nomogram being used prospectively to influence surgical decisions.[40] In that study, a pre-operative nomogram was used to decide whether or not intraoperative assessment of the tumour margins using frozen sections should be performed. In that study it was shown that using the nomogram to influence surgical decisions did not significantly increase the re-operation rate due to a positive resection margin compared with the control group. By using the nomogram, the surgical time of almost 62% of patients was reduced without any detrimental effects. While this study lends some support to the concept of using a pre-operative nomogram to improve surgical decisions for the benefit of the patient, additional studies are required to confirm the results of this single study.

There are many limitations to the nomograms developed in the current study that would limit their immediate application in clinical practice. These limitations can be divided into three main components, namely 1) the nomogram construction, 2) nomogram interpretation and 3) its clinical application.

The first limitation of the proposed nomograms is in the construction of the algorithm that resides behind the pictorial nomogram. The nomograms described in the current study were constructed using data from a retrospective study that assessed the outcome for dogs with STS that were surgically excised in first opinion practice. Ideally, the patient cohort used to derive the nomogram should be representative of the diseased population. As

was described in Chapter 3, there are significant differences in the surgical outcomes of dogs with STS operated in first opinion practice and dogs that are referred to a specialist centre. Due to these differences it is likely that a nomogram developed from a first opinion population may not be transferrable to a case that is being managed in a referral centre. The bias towards low grade tumours within this archive population may also impact on the transferability of this nomogram to a wider population. This bias may also explain why tumour grade was not utilised within any of the nomograms developed in the current study, even though grade is one of the most consistent and validated tumour characteristics to differentiate likely behaviour after surgery.[37]

It should also be considered that the quality of surgery performed in first opinion practice will also have an influence on the rates of tumour recurrence on which this data is based. Veterinarians working in first opinion surgery will have a range of surgical skills, and very few will have had any particular training in oncologic surgery. As has been discussed in previous chapters, the ability to maintain an appropriate *en bloc* resection margin about a STS requires considerable confidence and cognisance of anatomical features. Inexperienced surgeons may not have the confidence to maintain a consistent dissection plane about the entire circumference of a STS, particularly if it is large, more firmly fixed to the surrounding tissues, or located close to vital structures. As the dissection proceeds deeper into the tissues, there will be a desire to find a comfortable cleavage plane that allows the tumour to more easily elevate away from the tissues. The pseudocapsule that surrounds a STS can provide the inexperienced surgeon with this comfort zone, and allows

them to complete the difficult phase of the surgery successfully. However, by straying closer to the pseudocapsule and reactive zone of the STS, there is a risk that a higher proportion of microscopic tumour cells will have been left behind in the wound. Because the nomogram is founded on the hypothesis that elements of tumour biology are influential on the risks of tumour recurrence, this variance in surgical quality poses the challenge that recurrence of a STS in some cases was due to inadequate surgery rather than the effects of tumour biology. If this is true, the logistic regression equation on which the nomogram is based will be inaccurate, leading to an inappropriate estimation of risk. Ideally, the nomogram would need to be validated or reconstructed using data derived from a population of dogs operated by trained oncologic surgeons, to ensure this risk of bias is eliminated.

Another important limitation of the data quality on which the nomograms are constructed is the fact that information on cases within the study was collected retrospectively; this may result in recall bias or inaccuracy within the responses. Veterinarians completing the survey were reliant on clinical notes that had been written many years previously. This raises the possibility that some of the clinical information supplied about the tumour may be inaccurate. This deficiency could have an impact on the Clinical nomogram, which utilised a subjective description about the tumour in its algorithm. For example, the distinction of whether a tumour is “fixed” or “mobile” is subject to individual interpretation by the clinician. In the IHC nomogram, the variables used were less liable to misinterpretation, as it utilised more

objective or defined data such as age, mitotic rate and immunostaining characteristic of VEGF and decorin.

An additional deficiency of the current study was the small number of cases used to construct the nomogram. The small size of the population cohorts used in both the Clinical and the IHC nomogram will have a significant impact on the ability to detect statistical differences between the covariates selected for inclusion within the nomogram. Large studies that contain many hundreds of patients are more likely to detect subtle influencing characteristics within a population that may potentially be overlooked in a smaller study cohort. Most human studies where nomograms have been described and accepted within the clinical community have typically utilised sample sizes 10-100 times larger than that used in the current study.

The small sample size will also reduce the reliability of the regression calculations. When an outcome is binary - i.e. did tumour recurrence occur or not - then published guidelines for nomogram construction state that the number of recurrences should be greater than 10 times the number of predictors used in the calculation, to give an expected error rate of less than 10%.[28, 43] In both of the nomograms developed in this study, the small sample size made it impossible to have 10 times the number of events for each predictor. For example, in the Clinical_train dataset, three covariates were used in the calculation but there were only 27 dogs with tumour recurrences. This is less than the 30 events that would be the minimum recommended number. Within the IHC dataset, four covariates were used in the calculation but only 26 recurrences were present. For the current study,

reducing the number of variables within the nomogram was not feasible as it resulted in less differentiation between cases, and reduced accuracy. Ideally, considering the number of included variables, the nomograms would need to be developed using a disease population at least two or three times larger than the one used.

The covariates used in the nomograms were selected using the results of a multi-variable Cox proportional hazards model. Because of the small number of cases in the cohort, covariates were selected when significance was only 0.15, rather than a more conventional figure of 0.05. The use of 0.15 means that there is a 15% (almost 1 in 6) chance that the selected variable does not actually influence the outcome as suspected. In contrast, when using a p value of 0.05 this means that there is just a 5% (1 in 20) chance that the effect of the variable is simply due to chance. By broadening the inclusion of potentially relevant cases into a selected variable in this way, the accuracy of the nomogram will suffer. Because the selected variable may now lack sufficient distinguishing power, the nomogram may identify cases that are at risk of developing tumour recurrence, when they did not. This lack of accuracy will increase the number of false-positive results and explains the poor specificity of the nomograms developed in this study. The only way of overcoming this potential Type 1 error would be to increase the sample size of the study population so that there were sufficient cases within each variable. This would ensure that the proportion that developed the relevant outcome was then sufficient to meet the accepted 5% statistical threshold.

Another potential source of error that may limit the reliability of the nomogram is if any of the selected variables are likely to exert an influence on another. If the variables used in the nomogram are not truly independent of each other, then there are no additional benefits from including the additional characteristic in the algorithm. This dependence may also bias the selection of cases, as a case with one dependent variable is likely to gain an additional score on the nomogram from its related variable. In the IHC nomogram developed in the current study, the variables decorin, mitotic rate, and VEGF were identified by the Cox model as having an independent influence on outcome and were selected as characteristics to be used in the nomogram. In Chapter 4, we did not identify any obvious correlation between VEGF and decorin on Chi square analysis. However, at a physiological level, decorin is recognised as an important tumour suppressor.[44] It follows that reduced levels of decorin within a tumour will increase the availability of VEGF and other sequestered cytokines within the tumour microenvironment.[45] Possible variations in the proportions of VEGF isoforms within the tumour, the focus of study in Chapter 5, may also impact on the bioactivity of this important angiogenic protein within the tumour microenvironment. The varied bioavailability of these cytokines within the tumour microenvironmnent will likely have diverse consequences on the tumour, including influences on cellular metabolism, mitotic activity and the production of other, unmeasured molecules that may influence tumour progression. It follows that the true independence of VEGF, decorin and mitotic index cannot be assured, and it is likely that more sophisticated statistical tools would be required to analyse this further.

The second component of nomogram construction that requires examination is whether the performance of the algorithm is reliable, and relevant to the target population. The ultimate goal of a nomogram is to predict the outcome for an individual as accurately as possible. Calibration is therefore an essential step of nomogram development as it provides an objective measure of the ability of the nomogram to reliably discriminate patients based on the individual characteristics used in the model. The predictive accuracy of a nomogram is defined by the concordance index (*C*-index), which provides an objective measure of the difference between the predicted outcome and the actual outcome. By knowing the *C*-index, together with the 95% confidence interval, it is possible to gauge how reliable a particular nomogram will be. A *C*-index of 0.5 suggests the nomogram has no discriminating ability, with the prediction no better than a 50:50 chance - similar to a 'heads' or 'tails' outcome from a coin-flip. In the current study, the *C*-index for the IHC nomogram was 0.84, which suggests that the nomogram was able to discern a patient that would experience tumour recurrence from a patient that would not develop recurrence 84% of the time. However, the 95% confidence interval suggests the actual range may actually be between 76% and 93%.

The gold standard for nomogram calibration is to utilise an independent dataset i.e. one that is distinct from the population originally used to develop the nomogram.[5] In the current study, external validation was performed for the clinical nomogram by splitting the original dataset into two populations, with one set used for development and training of the model, and the other for external validation. The external validation dataset showed a disappointing discrimination ability, with a *C*-index of only 0.51, compared

to 0.71 that was described for the training cohort. This poor performance of the external validation raises serious questions about the reliability of the nomogram construction and would make it unsuited to use in a clinical setting. The reasons for this poor performance of the nomogram with the external dataset ultimately reflect the many limitations of the training dataset that have discussed in the previous section on nomogram construction.

It should also be noted that splitting the original population into two, as was performed in this current study, does not create a truly independent dataset. This is because the population used for the validation has ultimately been derived from the same study as the training dataset. The cases in the validation dataset are thus influenced by the same biases and limitations that affected the training dataset; these biases and limitations were outlined in the previous section. Ideally, external validation should be performed with a truly separate population. Although the author did have data from a previous study that had been derived from the population of dogs operated in first opinion practice,[30] the histological descriptions for each of these tumours was limited and a precise mitotic index and necrosis score was not available. Immunohistochemical staining of these cases had also not been performed. None of this data was therefore appropriate for use with the existing nomogram.

External validation of the IHC nomogram developed in the current study was not possible due to the small size of the original population cohort. Attempts to divide the database into a training and a validation cohort failed as there were insufficient events in each group to allow for adequate statistical

modelling. In this instance, validation relied completely on a statistical methodology called bootstrapping. Using this method, a validation dataset is created by randomly sampling cases from the original cohort. Due to random selection, it is possible that Patient A from the original cohort may be represented 3 times, whereas Patient B will not be represented at all. The nomogram is then applied to this bootstrap cohort, and the *C*-index calculated. This process is then repeated again and again - for the current study the number of repetitions was 200 – and the mean of the *C*-index of all 200 bootstrap samples derived. For the IHC nomogram, there was good agreement between the *C*-index of the original dataset and the mean *C*-index by internal validation, but because this is achieved by resampling of cases from the original dataset, such good agreement should not be unduly surprising. Therefore, confidence in the performance of the IHC nomogram will only be achieved when it has been validated against an external population.

The final component of nomogram development that requires scrutiny is whether it is ultimately suited for use in a clinical setting. The ultimate purpose of a nomogram is to provide a patient or clinician with a better prediction of outcome or optimal treatment choices compared to clinical judgment alone. Because there will always be limitations and possible errors with any predictive tool, it is important that users of the nomogram understand the potential deficiencies of the method. One important criterion that a successful nomogram must meet is whether it can outperform clinical judgement; if an experienced clinician can provide a prognosis for the patient with a reliability that is equivalent or better than the nomogram, then there is

little to be gained by using this tool. There have been several studies in human medicine that have attempted to address this question. In one study evaluating patients with prostate cancer, the performance of 22 different nomograms was compared to clinical judgment alone. Only 13 (59%) of the nomograms showed a predictive ability better than a human expert.[46] In a further experiment, clinicians were provided with concise summaries of the patient, and asked to predict both the 5-year recurrence-free probabilities for each patient, and also the potential for the disease to have spread beyond the prostate. The clinician's predictions were then compared with the outcome provided by the nomograms. This study showed that nomogram predictions of organ-confined disease were comparable to those provided by a clinician, with a *C*-index of 0.79 and 0.78. However, other studies have suggested that nomograms can significantly outperform human experts,[26] and may provide a patient with the necessary objectivity to support a particular treatment decision.[42] However, it is also important to recognise that there are no current methodologies that can predict patient outcome with perfect accuracy. Furthermore, while a well-constructed and validated nomogram may be considered an accurate and discriminating tool for predicting a particular outcome for a patient with cancer, it must be recognised this is simply an objective prediction that may be isolated from other clinical considerations that may be relevant to that patient. A nomogram cannot make treatment recommendations based on other patient characteristics or co-morbidities, or act as a surrogate for interactions between the veterinarian and the pet owner or client. They also do not provide definitive information

on symptomatic disease progression or the potential for complications associated with treatment.[42]

6.5 Conclusion

In conclusion, the results for this current study provides the first evidence in veterinary oncology to support a role for the nomogram to assist with predicting the outcome for patients after surgery for STS. From the evaluations performed, a nomogram that incorporates data from IHC interrogation of the tumour is more reliable than a nomogram that does not. However, while it is evident that nomograms may have the power to become an important component of decision making for the cancer patient, they will need to demonstrate robust reliability and accuracy if they are to completely supplant the insight and judgement of a clinical expert.

Evidence from this study suggests a nomogram could play an important role in helping to identify patients who either have no risk of recurrence after surgery, or who are liable to experience recurrence at some time in the future. These latter patients may choose to undergo additional therapy – either a wider surgical resection, radiation therapy or chemotherapy – to help reduce this risk of recurrence. It is also of interest that the nomograms developed in this study were able to predict this recurrence risk by using a combination of clinical and biological information derived from the patient and tumour details only, and were not reliant on information that would become revealed once a surgery has been performed, such as the extent of histologic margins. This supports the conclusions from previous chapters that suggest it may be

elements of tumour biology and not the surgical strategy that are influential in the prognosis of STS. This discovery lends support for using a nomogram, or other predictive tools to help determine the actual surgical margins required for an individual tumour, using information gained from a pre-operative interrogation of the tumour. Additional study will be required to ensure such a tool could be reliably and confidently incorporated into routine surgical planning.

6.6 References

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Chapter 7:

Targeting vascular endothelial growth factor as an adjuvant treatment for soft tissue sarcoma

7.1 Introduction

In previous chapters, it was shown that increased levels of vascular endothelial growth factor (VEGF) within a soft tissue sarcoma (STS) influenced tumour progression. Using immunohistochemistry, a STS with diffuse VEGF immunostaining was more likely to recur or cause death of the dog; this influence was independent of the surgical margins performed.

The reason why increased immunostaining of VEGF within a STS should be associated with an higher rate of tumour recurrence is unknown. In Chapter 4, it was speculated that detection of high VEGF may be a surrogate indicator for a STS that has a more permeable pseudocapsule that enables a wider migration of neoplastic cells into the surrounding tissues.[1] Alternatively, the high VEGF level may indicate that the tumour has evolved from a more hypoxic microenvironment.[2] Such a tumour may harbour a higher proportion of dormant tumour cells that are able to regenerate when favourable conditions return to the wound bed after healing is complete.[3] A further possibility is the extracellular matrix (ECM) surrounding a tumour that had diffuse VEGF immunostaining will also be rich in VEGF and other

angiogenic molecules. This residual tumour microenvironment within the wound bed may be more enabling of further oncogenesis than the residual wound of a tumour with low VEGF. Either or all of these possibilities may allow an individual tumour to be more able to re-grow following surgical resection.[4, 5]

If high VEGF promotes tumour recurrence and is associated with poorer survival, it follows that suppression of VEGF production could potentially slow tumour recurrence and progression. To test this hypothesis, thalidomide was used to suppress VEGF production within splenic haemangiosarcomas (HSA) in a series of dogs. Thalidomide was used as this has been shown to be a potent suppressor of angiogenesis through its effects on the expression of several genes influential on angiogenesis, particularly VEGF.[6]

Dogs with HSA were selected for this study rather than STS to allow the hypothesis to be tested in a timely and cost-effective manner. Soft tissue sarcoma can be slow to recur, and recurrence may not develop until up to 2 years after surgery in the majority of patients. Furthermore, local recurrence may occur in only 20% of patients. Therefore, to determine whether or not thalidomide could significantly reduce the incidence of local recurrence of STS by 50%, almost 500 patients would be required in each treatment group. Recruiting enough dogs into such a study would be difficult and would take many years to complete. Furthermore, due to the high numbers of dogs required, such a study would be very costly to perform. In contrast, virtually all dogs with splenic HSA will die due to tumour recurrence and almost 50% of patients will develop metastatic disease after splenectomy within 2-3

months.[7-11] This high rate of rapid recurrence greatly reduces the time required to complete the study. A power analysis suggested that as few as 10 dogs would be required if thalidomide increased the survival time from 60 days to 300 days. An additional benefit of using HSA as a model is that these neoplasms are common in dogs, representing between 12 to 21% of all mesenchymal malignancies, with an estimated incidence rate of 24/100,000 dogs per year.[8, 12-14]. While HSA do have some differences to STS, both neoplasms are mesenchymal in origin and have been shown to share some common molecular characteristics.[15, 16] This suggests that an effect of thalidomide on the progression of HSA could translate to a similar result being expected for STS.

The aim of the study described in this chapter was to compare survival times of dogs that received thalidomide to dogs that did not receive thalidomide after they had undergone splenectomy due to splenic HSA. The hypothesis was that thalidomide would significantly increase the survival time in treated patients.

7.2 Materials and methods

7.2.1 Patient inclusion: Treatment group

The inclusion criteria for dogs recruited to the treatment arm of this study included recovery after splenectomy, and a histological diagnosis of HSA. There were no exclusion criteria. Sections from all tumours were confirmed to be splenic HSA by a specialist veterinary pathologist using a combination of histology and immunohistochemistry (IHC). The criteria that enabled

positive identification of a HSA were positive immunostaining for CD31 and/or Factor VIII-related antigen. Immunostaining was performed at a commercial laboratory using previously validated techniques,[17, 18] as described below. The patient signalment was recorded, as well as body weight and the date of surgery.

Complete tumour staging was performed in all dogs at the commencement of the study.[11] (Table 7.1) This was achieved with computed tomographic imaging (CT) of the abdomen and thorax, with 1.5mm sections. No contrast agent was used. All dogs were sedated for the scan with medotomidine (0.005 mg/kg) and torbugesic (0.1mg/kg), administrated intravenously. The presence and location of any possible metastatic lesions was noted, but fine needle aspiration or biopsies were not performed. Sedation was reversed using atipamazole 0.001mg/kg, subcutaneously. After recovery from sedation, the dogs were discharged to their owners.

Table 7.1:
Stage Classification for canine HSA (from Wood et al, Prognosis for dogs with stage I or II splenic HSA treated by splenectomy alone: 32 cases (1991-1993). J Am Anim Hosp Assoc. 1998;34(5):417-21)[11]

Stage I	Primary tumour only
Stage II	Primary tumour with splenic rupture or lymph node involvement
Stage III	Primary tumour with splenic rupture or lymph node involvement and evidence of distance metastasis

Owners were required to contact the principle investigator every month in order to receive further thalidomide medication for their dogs. On each occasion, the owners provided information on their dog's current status including activity levels, and the presence of any possible side effects.

A full clinical examination, and repeat evaluations of the abdomen and thorax using a CT scan was repeated 3 months after commencing treatment, using the same sedation protocol outlined above.

In the event of the dog's death or euthanasia, the primary care veterinarian was asked to confirm the cause of death. A cosmetic post-mortem of both chest and abdominal cavities was performed, and samples of any secondary lesions were collected for histopathology if they were present.

7.2.2 Patient inclusion: Control group

Control cases were recruited using records from two commercial laboratory services in New Zealand: Gribbles Laboratory and New Zealand Veterinary Pathology (NZVP). Databases from each laboratory were searched to identify dogs with histologically confirmed splenic HSA diagnosed during the same time period as the treatment arm of the study (2012-2014). Histology sections from identified tumours were reviewed by a specialist veterinary pathologist to confirm the diagnosis of splenic HSA, using a combination of histology and immunohistochemistry for CD31 and FVIII, as described below.

The veterinarians who submitted the samples were sent a standardised questionnaire requesting signalment information about the dog, as well as

details about the history, clinical examination and methods of tumour stage determination at the time of surgery. The current status of the dog at the time of questionnaire was also determined. In the event of the dog's death or euthanasia, the veterinarians were asked for information relating to the cause of death, in particular if this was considered due to the HSA. Dogs were included in the control group if the following criteria were met: complete splenectomy was performed, no adjuvant treatment had been provided for the HSA, adequate clinical notes were available for review, and the current status of the dog, including cause of death, was known.

7.2.3 Immunohistochemistry

Immunohistochemistry was performed on 4μm sections of formalin fixed paraffin embedded tissue sections that were cut onto positively charged slides for each neoplasm. Each slide was deparaffinised and rinsed onboard a Benchmark Ultra staining platform (Ventana Medical Systems) and processed as follows for CD31 (PECAM-1) and von Willebrands factor (FVIII) immunohistochemical staining.

CD31: The slide was heated to 100°C and incubated with cell conditioner 1 for 32 minutes to retrieve epitopes. The slide was then rinsed and taken to 36°C; a peroxidase inhibitor was applied and incubated for 4 minutes. Next, 100μL of 1:200 dilution of mouse antihuman CD31 monoclonal antibody (CD31 (JC70A): Dako Australia Pty. Ltd), diluted in antibody diluent (Ventana), was applied and incubated at 36°C for 8 minutes. Visualisation was achieved via the Ventana Optiview Detection Kit. Secondary antibody was applied and

incubated for 8 minutes, then hydrogen peroxide and DAB for 8 minutes. Copper was applied for 4 minutes to stop the reaction.

von Willebrand Factor (FVIII): The slide was heated to 36°C, a peroxidase inhibitor was applied and incubated for 4 minutes. Next, 100µL of Protease 1 (Ventana) was applied and incubated for 8 minutes to retrieve epitopes. Next, 100µL of a 1:400 dilution of rabbit anti-human von Willebrand Factor (FVIII (P0226), Dako Australia Pty. Ltd), diluted in antibody diluent (Ventana), was applied and incubated for 16 minutes at 35°C. Visualisation was achieved via the Ventana Ultraview Detection Kit. Secondary antibody was applied and incubated for 8 minutes, then hydrogen peroxide and DAB for 8 minutes. Copper was then applied for 4 minutes to stop the reaction.

All sections were counterstained with Gills Haematoxylin (Surgipath). Internal canine control tissues (vascular endothelium) were present on all slides

7.2.4 Thalidomide preparation

Thalidomide (α-N- [phthalimido] glutarimide, $C_{13}H_{10}N_2O_4$), was prepared at Massey University using a two-step process as previously described.[19] Briefly, glutamine was reacted with N-carbethoxyphthalimide in water in the presence of sodium carbonate (99% Riedel de Hahn, water free) at room temperature. Then, the product of this reaction (N-phthaloyl-L-glutamine) was reacted at reflux with 1,1'-carbonyldiimidazole in the presence of a catalytic amount of N,N-dimethylaminopyridine in tetrahydrofuran. The two procedures were followed exactly as previously described,[19] with the only

alteration being that the first step was based on 100 grams of N-carbethoxyphthalimide and the second step was based on 100 grams of carbonyldiimidazole. All yields and analytical data were consistent with those previously reported, giving confidence for drug purity.[19] Confirmation of 99.7% synthesis purity was performed by nuclear magnetic resonance (600MHz) analysis, with a sample of pharmaceutical-grade thalidomide used as a reference material.[20]

The dog owners were asked to read and sign a consent form indicating they understood the potential human health implications of thalidomide exposure, and women of child-bearing age were advised not to handle the drug. Non-sterile latex gloves were supplied to the owners to be worn at the time of drug administration, with recommendations for hand washing after handling thalidomide. Each client was provided with a lockable box for storage of the thalidomide, to ensure the drug could not be accidentally handled by other people.

Following the initial evaluation, all patients in the treatment arm of this study received thalidomide with treatment commencing on the evening after CT examination. Owners were encouraged to give the thalidomide to their dogs in the evening, due to the known somnolence effects of this drug.[6]

All dog owners were provided with a 30-day supply of thalidomide at a time; this restricted supply was utilised to minimise the potential wastage of drug supply should the dog die, and also to ensure that monthly updates about the dog's general health were received from the owners. All dogs received daily administration of the drug from the time of their initial examination until

their death. Thalidomide was dispensed into individual gel capsules at a previously recommended dose of 8.5mg/kg *per os* once daily.[21] Each 30-day batch of capsules was prepared from the bulk supply of thalidomide, which was cool-stored in a sealed container. Capsules were individually weighed during preparation, with a 10% tolerance for the actual dispensed weight of the capsule compared to the desired dose.

7.2.5 Statistical Analysis

Analyses were performed with statistical software (SPSS Statistics v24.0.0, IBM, New York, NY). Death or euthanasia due to the HSA was the primary end point for the study. Survival times (ST) were calculated from the date of surgery until the date of death. For survival calculations, dogs that died because of their tumour were considered completed events. Dogs that were still alive at the time of the study were censored at the close of the study.

Survival time was calculated from the date of splenectomy (T0) to death or censor date. The interval from when splenectomy was performed to the start of thalidomide therapy was defined as the treatment gap.

The Kaplan-Meier method was used to analyse ST according to age, sex, neuter status, tumour stage. Cox regression analysis was performed to assess the impact of treatment gap on outcome. $P < 0.05$ was considered significant.

The study was approved by the Massey University Animal Ethics Committee (application MU 11/56).

7.2.6 Historical population

A literature search was performed in PubMed (NCBI, Bethesda, USA) to identify all previous published studies for dogs where splenectomy had been performed to manage splenic haemangiosarcoma. Data retrieved from each article included: the number of animals in each treatment group; tumour stage at the time of treatment (if known); the details of any adjuvant treatments used; overall median survival time and range. This data was tabulated.

7.3 Results

7.3.1 Patient characteristics

Patient and tumour characteristics of both cohorts are summarised in Table 7.2. From August 2012 to December 2014, fifteen dogs were recruited into the thalidomide treatment group. The age of the dogs ranged from 7 to 14 years, with a median age of 10 years. Eight dogs were male, and 7 were female. A variety of breeds were represented including German shepherd (4), Labrador retriever (3), heeler, boxer, dachshund, elkhound, border collie, huntaway, miniature schnauzer, and a Welsh corgi. Sixteen dogs were included in the control group. The age of the dogs ranged from 6 to 15 years, with a median age of 10 years. Eleven dogs were male and 5 were female. Dog breeds represented included border collie (3), German shepherd (3), golden retriever (2), Labrador retriever (2), beagle, great Dane, Jack Russell terrier, miniature schnauzer, Staffordshire bull terrier and a Tibetan spaniel.

There were no significant differences in the age or sex of dogs between treatment and control groups.

Table 7.2
Patient and tumour characteristics of treatment and control cohorts

Characteristic	Treatment (n=15)	Control (n=16)
Age	7-14 (median 10 years)	6-15 (median 10 years)
Sex Female Male	 7 8	 5 11
Tumour presentation	all haemoperitoneum	all haemoperitoneum
Stage Stage 1 Stage 2 Stage 3 Unknown	 0 10 5 0	 0 9 4 3

7.3.2 Neoplasm staging

In the treatment group, 5 dogs were classified as stage 3 due to the presence of cystic lesions within the liver on CT scan. No concurrent thoracic or cardiac lesions were identified in any dog. The suspected metastatic lesions ranged in size from isolated clusters of 4mm radiolucent cysts to a large 4cm multi-loculated cystic mass within the left lateral lobe. A fifth dog was classified as stage 3 due to a histological diagnosis of metastatic lesions within the omentum. The remaining 10 dogs were classified as stage 2, as no evidence of metastatic lesions was identified in any abdominal or thoracic organ.

Within the control dogs, there was more variability in the methods used to stage the HSA. In 5 dogs, thoracic radiographs and abdominal ultrasound were performed prior to surgery while in 8 dogs, thoracic radiographs were performed prior to surgery, and the liver and other abdominal organs were visually inspected at the time of surgery. In the remaining 3 dogs, no thoracic or abdominal imaging was performed prior to surgery, but the abdominal organs were visually inspected at the time of surgery. Based on the information provided by the referring veterinarians from these investigations, four dogs in the control group were classified as stage 3 due to the presence of metastatic lesions within the liver (3 dogs) or omentum (1 dog). A further 9 dogs were classified as stage 2. The stage at the time of surgery could not be reliably defined in 3 dogs as thoracic radiographs had not been obtained prior to surgery.

7.3.3 Patient outcomes

The follow-up period ranged from 6-660 days (median 90 days). Results are summarised in Table 7.3.

Thirty of the 31 dogs enrolled in the study died during the study period. This included 14 of the 15 dogs in the treatment group and all 16 control group dogs. A diagnosis of haemoabdomen due to bleeding from one or multiple metastatic lesions in the abdomen was confirmed at necropsy in 13 dogs in the treatment group. In another dog in the treatment group, extensive bleeding isolated to the retroperitoneum was identified at post-mortem, with histologic evidence of HSA in the adjacent kidney. One other dog in the treatment group was euthanatised 628 days after surgery due to progressive

senile behaviour and separation anxiety. No evidence of neoplasm recurrence had been observed in a CT of the chest and abdomen on the day of euthanasia; no necropsy was performed. One dog remained alive at the end of study period, 594 days after surgery. All dogs in the control group died or were euthanased due to acute weakness or collapse. Specific details about the clinical findings at the time of euthanasia were not consistently provided by veterinarians, and was only available for 9 of the 16 dogs. For these 9 dogs, anaemia was recorded in 7 cases, with recurrence of a haemoabdomen confirmed by abdominocentesis in 5 cases.

Table 7.3:
Statistical analysis of outcomes for treatment and control groups

Treatment Group	Stage 2	Stage 3	Overall	p value
Thalidomide				
n= Mean survival time Median survival time	10 389 days 303 (0-744)	5 40 days (31-48)	15 279 days 172 days (93-250)	p<0.0001
Control				
n= Mean survival time Median survival time	9 87 days 49 days (0-98)	4 20 days 13 days (0-36)	16 67 days 32 days (26-37)	p=0.03
	p=0.001	p=0.001	p=0.01	

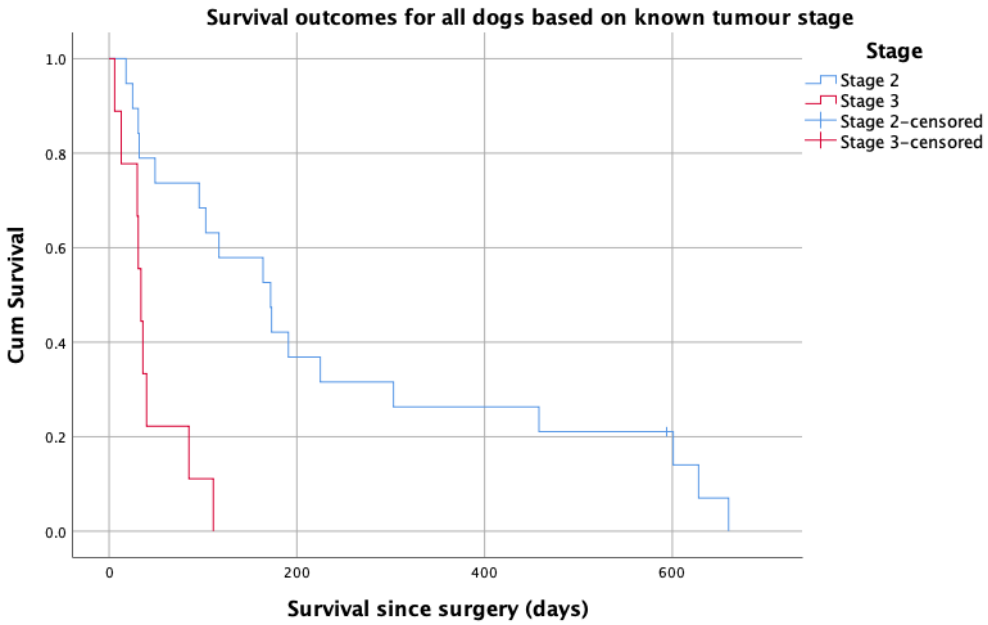
7.3.4 Median survival times

7.3.4.1 Effect of tumour stage: all dogs

For all 28 dogs enrolled in the study for which tumour stage could be determined, tumour stage was found to significantly influence survival. Dogs

with stage 2 tumours had a MST of 172 days compared to 34 days for dogs with stage 3 HSAs (p = 0.0003) (Figure 7.1).

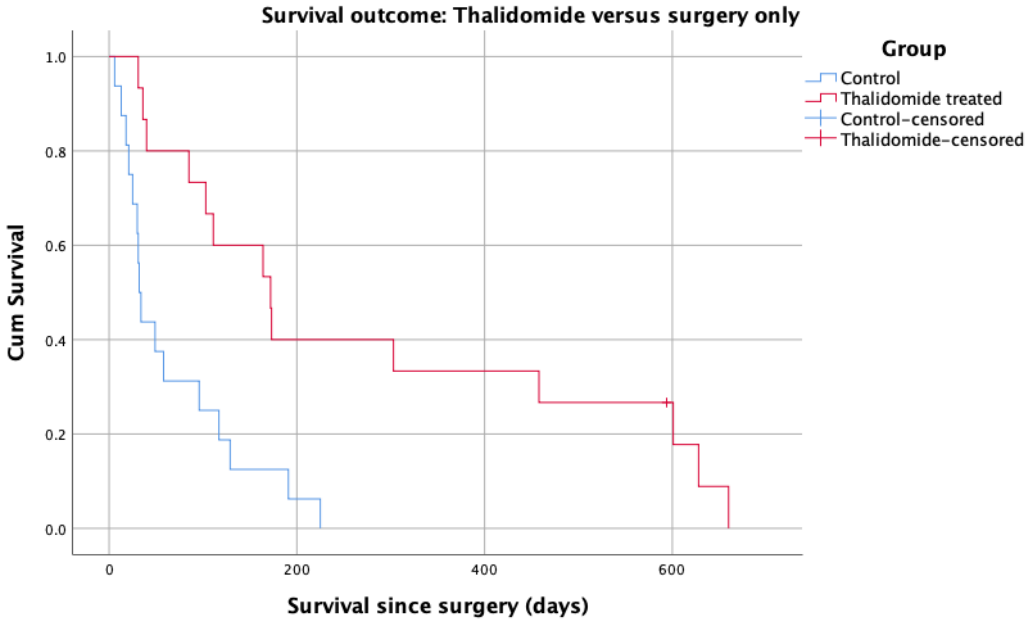
Figure 7.1:
Kaplan Meier survival curve showing differences in survival based on tumour stage for all dogs enrolled in this study



7.3.5 Effect of thalidomide treatment

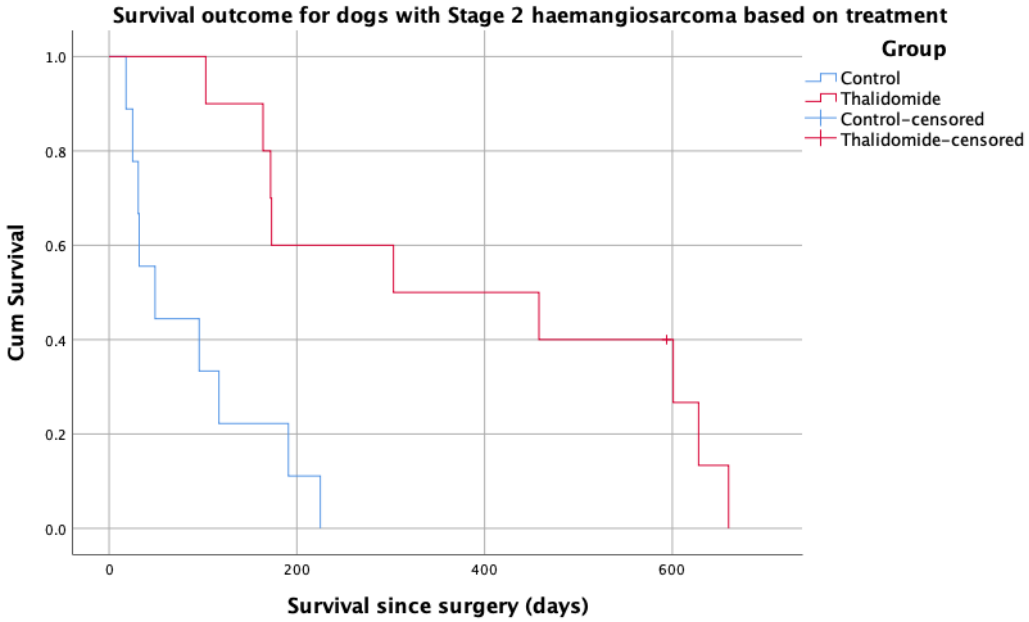
Dogs receiving thalidomide survived between 31 and 660 days and had a median survival time (MST) of 172 days (95% CI 93-250 days). In comparison, dogs in the control group survived between 6 and 225 days, with a MST of 32 days (95% CI 26-37 days). The use of thalidomide significantly increased the survival times for dogs receiving treatment (p=0.001) (Figure 7.2).

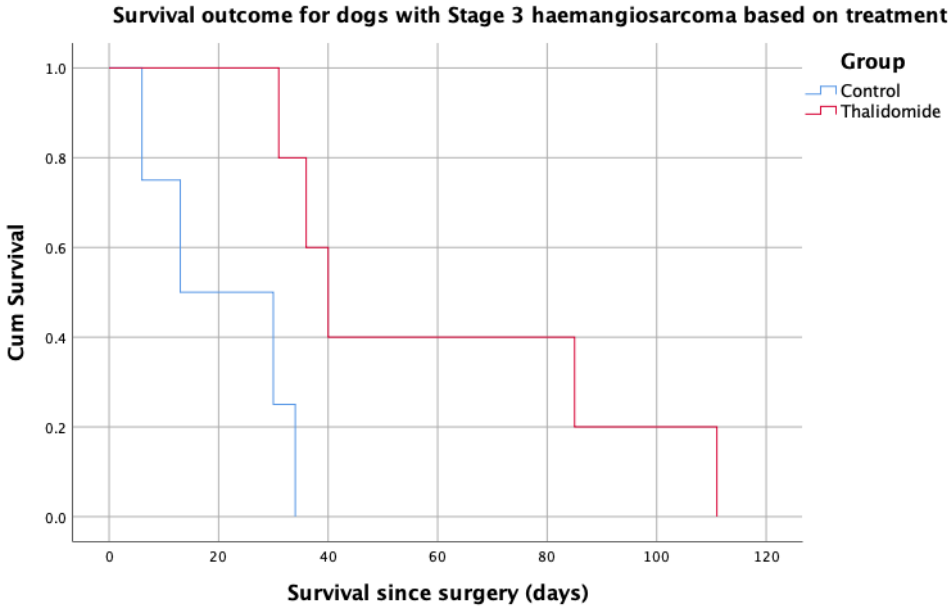
Figure 7.2:
Kaplan Meier survival curve showing differences in survival based on tumour stage for dogs receiving thalidomide compared to the control population



For dogs with stage 2 disease, thalidomide treatment improved the MST over the control group from 49 days to 303 days, giving a hazard ratio (HR) for death in the treatment group of 0.2 (95% CI, 0.08 to 0.63; P=0.005; Figure 7.3a). For stage 3 disease, a significant difference in survival was also noted, but the survival advantage was less pronounced (13 days vs 40 days, p=0.01; Figure 7.3b). The HR for death in stage 3 dogs receiving thalidomide was 0.09 (95% CI, 0.01 to 0.85; P=0.04).

Figure 7.3a and 7.3b:
Kaplan Meier survival curve showing differences in survival based on tumour stage for dogs receiving thalidomide compared to the control population





When the effect of tumour stage for dogs within each treatment group were considered separately, significant differences in survival times were also observed. Treated dogs with stage 2 tumours had a MST of 303 days (95% CI

0-744 days) which was significantly longer than the 40 days (95% CI 31-48) seen in dogs with grade 3 tumours (Log Rank 15.7, p<0.0001). Untreated dogs with stage 2 tumours had a MST of 49 days compared to just 13 days (95% CI 0-36) for stage 3 dogs (Log Rank 4.6, p=0.03).

7.3.6 Effect of treatment delay

The median time to start thalidomide treatment was 11 days after surgery. This delay was primarily due to the time taken to obtain histologic confirmation and subsequent examination for staging. Two dogs did not start treatment until more than 30 days after surgery, and one dog did not start until 62 days. However, there was no significant influence of this treatment delay on outcome (HR 1.010, 95% CI 0.97 - 1.049, p=0.59).

7.3.7 Medication compliance and side-effects

All dogs were reported to receive their daily dose of thalidomide continuously until their death once treatment was started. There were no disruptions due to logistical problems with delivery of medications or adjustment to the dose or dosing interval due to side-effects.

All owners commented on some sedation within 30 minutes after drug administration, but in no dog was this of sufficient concern to warrant a change in the administered dose. One dog, the lightest in the study at 6.5kg, was reportedly mildly stuporous for the first few days after medication commenced, but these effects resolved by the end of the first week with no change in treatment dose. One working police dog and one working Guide

Dog remained in full work while on thalidomide, with no apparent change in their concentration abilities or working routines.

No other side-effects were reported while dogs were receiving thalidomide medication.

7.3.8 Historical population

There were 19 previous studies published that described the outcome of dogs after treatment for splenic HSA by splenectomy. In 14 of these studies, some form of adjuvant therapy was provided following surgery. The average median survival time for dogs that underwent splenectomy only was 52 days (range 19-86 days). The average median survival time for dogs that also received some form of adjuvant therapy was 152 days (range 91-273 days) (Table 7.4).

Table 7.4:

Statistical analysis of outcomes for all dogs receiving thalidomide compared to previously published studies and a contemporaneous control population

Study	Adjuvant treatments	Group (n=)	Median Survival Time (days)
Thalidomide	+ Thalidomide	15	172 (31-744)
Contemporaneous control (this study)	Surgery only	16	32 (6 – 225)
Johnson et al 1989	Surgery only	19	56
Brown et al 1985	Surgery only	21	65
Wood et al 1998	Surgery only	32	86 (14-470)
Prymak et al 1988	Surgery only	59	19
Kim et al, 2007	Surgery only	41	87 (14-790)
Brown et al, 1985	+ Mixed Bacterial Vaccine	10 (spleen)	91
Brown et al, 1985	+ Mixed Bacterial Vaccine & vincristine/doxorubicin/cyclophosphamide	10 (spleen)	117
Dervisis et al, 2011	+ Doxorubicin/darcarbazine/vincristine	19 (all types)	125 (18-411)
Finotello et al, 2015	+ Doxorubicin/darcarbazine	15 (spleen)	140
Finotello et al, 2015	+ Doxorubicin/cyclophosphamide	5 (spleen)	142
Gardner et al 2015	+ Doxorubicin/toceranib	31 (spleen)	172 (range NR)
Hammer et al	+ Vincristine/doxorubicin/cyclophosphamide/chlorambucil/methotrexate	6 (spleen)	145
Johnson et al, 1989	+ Vincristine/doxorubicin/cyclophosphamide	3 (spleen)	140
Kahn et al, 2013	+ Doxorubicin/deracoxib	21 (spleen)	150 (21-1506)
Sorenmo et al	+ Doxorubicin/cyclophosphamide	6 (spleen)	180
Vail et al, 1995	+ Doxorubicin/cyclophosphamide	16 (spleen)	141
Vail et al, 1995	+ Doxorubicin/cyclophosphamide & L-MTP-PE	16 (spleen)	273
Kim et al, 2007	+ Epirubucin	18 (spleen)	144 (74 -2717)

7.4 Discussion

The present study showed that thalidomide may prolong survival of dogs with splenic HSA. To the author's knowledge, this was the first prospective study to assess the effect of adjuvant thalidomide on patient outcome after splenectomy for HSA. Previously published studies had suggested that good patient response was possible with this drug,[9, 21] but these trials were stopped as thalidomide became unaffordable later in the studies. The results of the present study confirm the potential of thalidomide as a useful adjuvant therapy for canine splenic HSA. It also provides evidence to support the investigation of thalidomide as an adjuvant treatment for other mesenchymal neoplasms including STS, which were the focus of this thesis.

In this study, the overall survival times of dogs receiving thalidomide after splenectomy were improved when compared to dogs treated by splenectomy alone. The most beneficial effects of thalidomide were observed in dogs with stage 2 HSA. Within this stage group, thalidomide treatment improved the MST over the control group from 49 days to 303 days. Although there was a significant survival advantage noted between the thalidomide and control dogs with stage 3 HSA, the survival benefit was smaller with the MST only increasing from 13 days to 40 days. The key difference between these stages is the metastatic lesions in stage 2 HSA are not grossly discernible using existing imaging modalities and may range in size from individual cells to a small nodule <1-2mm in size. In stage 3 dogs, the metastatic lesions may range from just a few millimeters to masses many centimetres in size; these larger lesions tend to be very fragile and prone to rupture. The reduced

survival time for stage 3 dogs is therefore understandable, because the gross metastatic lesions present in these animals will be more susceptible to spontaneous rupture leading to internal haemorrhage. Thalidomide treatment did appear to remain effective in this group; the HR for death in the treatment group was 0.1, which suggests that stage 3 dogs receiving treatment had a 90% lesser chance of dying from their HSA for every day they received thalidomide when compared to dogs of an equivalent tumour stage who were not receiving the drug. However, the confidence interval of this HR was wide, meaning the survival benefit could have been anything between 15% and 99%. The small number of dogs in this group makes further analysis of the possible benefit of thalidomide in this treatment stage difficult.

It is well documented that the prognosis for dogs with splenic HSA is consistently poor.[22] While surgery is the standard therapy for HSA,[23] this is considered palliative as metastasis has usually occurred by the time of surgery.[9] As a consequence, almost all dogs will die from secondary tumours within 1 year of surgery unless additional treatment is given.[9, 11] In the current study, five of the dogs that received thalidomide – representing almost one-third of the study population – survived more than one year after surgery. The results of this study provide sufficient evidence to support further investigation comparing thalidomide alongside other adjuvant chemotherapy strategies suggested for canine HSA.

The ability of adjuvant chemotherapy to increase the survival times of dogs with splenic HSA has been previously demonstrated, but despite the use of

various treatment combinations the duration of remission with most protocols remains short.[9, 13, 23-27] Current adjuvant chemotherapeutic protocols have utilised doxorubicin alone, doxorubicin with cyclophosphamide, vincristine with cyclophosphamide and doxorubicin, dacarbazine and vincristine; no individual protocol has been shown to be superior. When doxorubicin-based protocols are used, studies have reported median survival times of 125-180 days.[24, 25, 28-31] By comparison, thalidomide resulted in an equivalent median survival time of 172 days. While these results suggest that thalidomide could be as effective as conventional cytotoxic medications, it is disappointing that the overall survival for HSA was not improved beyond our current expectations.

The results from the current study suggests that the targeting of angiogenesis alone may actually be insufficient to prevent tumour progression.[32, 33]. Almost all dogs in the treatment group died as a consequence of haemorrhage from a ruptured secondary tumour, which suggests that thalidomide was unable to prevent the development of metastatic disease. It may be possible that combining an anti-angiogenic treatment like thalidomide with conventional cytotoxic drugs could provide some synergistic benefits. Using this strategy, conventional cytotoxic drugs would act to kill residual HSA by directly targeting the cancer cells, whilst thalidomide would be used to disrupt crucial angiogenic pathways, and inhibit the reactivation of dormant tumour clones and other resistant populations. Other authors have examined the outcomes when conventional cytotoxic medications are combined with anti-angiogenic or tumour environmental modulators. In one study, dogs with HSA were initially

treated with conventional chemotherapy using a doxorubicin-based protocol.[29] After completion of this treatment course, a subset of dogs then received continuous treatment with a small molecule inhibitor (Toceranib phosphate, [Palladia®]) that has activity against VEGF and PDGF. None of dogs treated with the small molecule inhibitor therapy showed any improvement in disease-free interval or overall survival. However, the apparent lack of response to toceranib may have been due its delayed administration, which was not started until a full-course doxorubicin chemotherapy was completed, or due to a more limited angiogenic effect of Palladia compared to other drugs like thalidomide. It would be useful to repeat this study, but using a combination of conventional cytotoxic chemotherapy in conjunction with thalidomide to determine if there is any possible synergistic effect.

In recent studies, thalidomide has also been combined with other anti-angiogenic protocols, including metronomic chemotherapy (MTC).[34] Metronomic chemotherapy, also called continuous low-dose chemotherapy, uses a combination of cyclophosphamide and a cox-2 selective non-steroidal anti-inflammatory drug (NSAID). Metronomic chemotherapy is considered to disrupt tumour progression via two key mechanisms: firstly, by disrupting host endothelial cells from dividing and forming new vasculature and secondly, by restoring the innate anti-tumour immune response by depleting regulatory T cells (Treg) and restoring the natural killer (NK) and T cell functions within the tumour microenvironment.[35-37] Metronomic chemotherapy alone has been reported to achieve comparable outcomes to historical survival times for dogs treated with conventional cytotoxic

chemotherapy.[35] A synergy between cyclophosphamide and thalidomide has been demonstrated in a mouse model,[38] so it is reasonable to assume that combining these drugs could lead to further improvements in tumour control. A recent study has reported results when thalidomide was included with a MTC protocol.[34] Unfortunately, this was a multi-institutional retrospective study, so the chemotherapy protocols were not consistent for all dogs. This study was also limited to the treatment of dogs with stage 3 HSA only. Nevertheless, 87% of the dogs that received MTC also received thalidomide at a dose of 2-4mg/kg *per os* once daily. Sadly, the apparent benefits of MTC plus thalidomide in this study were disappointing with the survival time for dogs receiving conventional chemotherapy being 140 days, which was significantly better than the survival times for dogs that were receiving MTC plus thalidomide (58 days). Because this study was limited to dogs with stage 3 HSA, the potential synergy that may be gained from combining thalidomide and MTC for all dogs with HSA remains unknown. However, the results of this study do suggest that anti-angiogenic treatment may provide only a limited survival benefit for dogs with gross metastatic lesions, which is consistent with the results of the current study. It is possible that anti-angiogenic treatment alone may be less effective on macroscopic tumours, perhaps because the angiogenic switch becomes self-sustaining beyond a certain point in tumourigenesis. It may be that the antiangiogenic and immunomodulatory effects of drugs such as thalidomide and cyclophosphamide can no longer disrupt the progression of a tumour once it has established in its own microenvironment.

Thalidomide was chosen for the current study due to its reported effects on suppression of VEGF. The previous studies documented in this thesis had determined that STS with an increased immunostaining of VEGF were associated with higher rates of local recurrence and reduced survival; it was therefore reasoned that VEGF could be an appropriate therapeutic target. However, the potential benefits of thalidomide are not limited to VEGF alone, and this drug is known to have a range of diverse effects on the regulation of angiogenesis, as well as immunoregulatory and inflammatory pathways.[6, 39-47] For example, thalidomide has been shown to induce the down-regulation of several inflammatory cytokines including TGF-β and NF-κB;[40] increased expression of these pathways has been shown to be significant indicators for reduced disease-free survival in human STS.[16] Thalidomide is also known to stimulate primary T lymphocytes to increase their anticancer activity. In canine STS, levels of regulatory $CD4^{+}Foxp3^{+}$ regulatory T cells (Treg) are increased,[48] which can cause inhibition of innate anti-tumour immune responses. Recent studies have shown that thalidomide and cyclophosphamide can act to eliminate these suppressive Treg cells,[48, 49] thus restoring the normal immune response to the tumour. In further studies, it would be helpful to evaluate the status of these other treatment pathways, to determine if there is a critical tumour size at which point tumour growth can no longer be impeded by anti-angiogenic treatments such as thalidomide.

Despite the positive results reported in the current study, the cost of thalidomide may limit the potential for this drug becoming accepted for routine use into veterinary treatment. Even for humans, the cost of

thalidomide for patients has become increasingly controversial as the clinical indications for its use have expanded.[50] Thalidomide is a drug with a terribly legacy and these historical issues continue to impact on its availability.[51] For humans, access to the drug remains rigidly controlled and prescription is only possible if the patient agrees to comply with a System for Thalidomide Education and Prescribing Safety (STEPS). The conditions required under this program include limiting prescription and dispensing rights to authorised prescribers and pharmacies, keeping a registry of all patients prescribed thalidomide, providing extensive patient education about the risks associated with the drug, and providing periodic pregnancy tests for women who take the drug.[52] Under normal circumstances thalidomide would now be off-patent, and cheaper generic derivatives of this drug would be available. However, because of the rigidly enforced legislation that surrounds thalidomide there is still only one manufacturer with the licence to bring the drug to the market. By citing the restrictive legislative agreements, this company has reportedly actively inhibited the development of generic alternatives.[53] However, since 2000, the price of thalidomide has increased by almost 1000%.[50] This price inflation has impacted on veterinary use of this drug; initial clinical trials that started in the late 1990s were abandoned due to the rising cost of the drug.[9, 21] Based on 2019 prices for thalidomide from veterinary suppliers in the UK, the monthly cost of thalidomide for a 30kg dog would be more than £1200. Given that thalidomide needs to be given continuously for the remainder of a dog's life, this could equate to an annual cost of almost £15,000. This cost would exceed the ability for many pet owners to afford therapy. This is unfortunate as, based on the results of

the current study, thalidomide may prove to be an important drug in the management of canine HSA and potentially other mesenchymal tumours.

The optimal therapeutic dose of thalidomide has not been well-established in the dog. Aside from a specific toxicity study, where doses ranging from 43-1000mg/kg/day were used, various clinical reports have used doses ranging from 2-3mg/kg q24h or q48h,[34] to 20mg/kg/day.[54] The dose used in the current study was based on recommendations from the authors of a discontinued trial of thalidomide in canine HSA, where some beneficial effects had been noted.[21] Further study may be required to help determine the optimal therapeutic dose in the dog. One recent publication has described a gradually reducing dose strategy when thalidomide was used to treat malignant cancer affecting the mammary gland of dogs. In that study, thalidomide was initially given at 20 mg/kg once daily for 3 months, after which time the dose was reduced to 10 mg/kg once daily until the patient's death from the tumour. This study reported improved survival times for dogs with advanced stage disease compared to those receiving conventional chemotherapy only.[54] Treatment was apparently well-tolerated, although excessive somnolence was reported at the 20mg/kg dose in some patients. It is likely the cost of thalidomide may lead a drive to determine efficacy at the lowest possible dose in the dog. Even in humans, the optimal dosing schedule for thalidomide is unclear, with doses of between 200 – 800 mg/day used in most cancer trials.[55] For people, the dose is usually titrated to the highest dose tolerated by the patient with the minimum of side effects. It is not clear if there is a dose-response relationship, or if smaller doses are equally effective with lesser side effects.

Apart from drowsiness and the well-known teratogenic effects, chronic use of thalidomide tends to be well tolerated.[56] For that reason, monitoring for haematologic or biochemical toxicities was not performed in this study. Human patients have experienced peripheral neurological disturbances such as hypo- and hyperalgesia, impaired temperature sensitivity, and polyneuritis, but these effects have not been observed in the dog.[57] Other less commonly reported side effects include constipation, deep vein thrombosis and a mild skin rash.[56] In toxicity studies performed in dogs, no evidence for systemic toxicity was reported with thalidomide doses ranging from 10 - 1000 mg/kg/day.[54, 57]

A major limitation of this study was there was no placebo or alternative treatment arm included in the clinical trial design. No placebo treatment was included in this study because it is already well-established that the outcome for dogs with HSA treated with surgery alone is poor; median survival times of just a few months have remained unchanged in publications over the last thirty years. The outcome for patients receiving thalidomide was therefore compared with a second population of dogs with HSA that had been treated by surgery alone during the same time period. While this provided a contemporaneous control population with which to compare outcomes, this decision resulted in a different quality of staging investigation for the HSAs between the treatment and the control groups. All of the dogs receiving thalidomide had a CT scan of the chest and abdomen at the start of the study, and again after 3 months of treatment. By comparison, none of the dogs in the control population underwent CT staging, and only 5 of 16 dogs had an ultrasound evaluation of the liver, which was the most common site for

metastasis in the thalidomide group. It is likely that the plain thoracic radiographs and visual inspection of the external surface of the liver, which represented the full extent of staging investigations for most dogs in the control group, may have failed to detect many small or deep parenchymal lesions. It is therefore possible that some dogs within the control group had undetected metastases at the time of splenectomy. This would effectively increase the proportion of stage 3 dogs within the control population, and impact on overall survival figures. Therefore, when comparing the outcome for dogs in this study, this difference in the quality of staging investigation could effectively bias the results in favour of thalidomide. While such a bias cannot be excluded, it is reassuring to note that the survival times observed for the dogs in the thalidomide group was notably superior to that of dogs receiving no therapy in all previously published studies;[9, 13, 24-27] this provides some confidence in the ultimate conclusion of this study. This limitation could have been overcome by incorporating a placebo arm, which would ensure that all dogs in the study underwent the same staging evaluation by CT, whilst only 50% of the dogs received medication containing the active ingredient. However, this would have doubled the number of CT scans required and increased the overall cost of the study. Also, because initial data on thalidomide in HSA suggested a beneficial response was likely,[21] the author could not, with good conscience, deny patients a treatment that carried a reasonable chance of improving their survival. An alternative strategy would have been to increase the number of dogs in the contemporaneous control population, so that any potential differences in

staging evaluation between the two groups became less influential in the analysis.

A second limitation of the current study is the potential for bias that can occur when comparing the outcome of the treatment group with historical literature, or with a population of dogs randomly sourced from a different sector of the population. Because these dogs may have owners derived from a different financial or social stratum than the dogs enrolled in the study, their outcomes may be affected by extraneous factors other than the treatment or disease. Furthermore, these owners may have been less inclined to delay euthanasia as they did not have the same emotional commitment to the objectives of the study. However, this potential bias is largely overcome in the current study because all but one dog died due to the development of spontaneous bleeding into the abdomen as a result of progression of metastatic disease. The decision for euthanasia was therefore influenced directly by disease progression, rather than the emotional commitment of the owner.

7.5 Conclusion

From the results of the current study, thalidomide appears to be well-tolerated clinically and may significantly improve survival for dogs following surgery for splenic HSA. Tumour stage at the start of treatment may be important; thalidomide showed improved survival benefits in dogs that had no visible metastatic lesions when starting treatment. From these results, the

use of thalidomide should be considered as a potential adjuvant treatment for dogs with HSA, and for other mesenchymal tumours such as STS.

In order to evaluate whether thalidomide treatment had any effect on the presence of VEGF within tumour, the next study describes an immunohistochemical study that compares the metastatic lesions of dogs that received thalidomide compared to group of dogs that did not.

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Chapter 8:

Analysis of the effect of thalidomide on vascular endothelial growth factor in the metastatic lesions of treated patients

8.1 Introduction

In the previous chapter, it was determined that survival times for dogs with splenic haemangiosarcoma (HSA) were significantly lengthened by treatment with thalidomide following splenectomy. Thalidomide is known to be a potent suppressor of vascular endothelial growth factor (VEGF) production, as well as other cytokines of importance in tumour angiogenesis.[1-3] Although the study described in the previous chapter showed that thalidomide significantly improved the overall survival for dogs with HSA, dogs treated with thalidomide still developed metastatic disease which resulted in the death of the dog. This suggests that the tumour can continue to stimulate angiogenesis independently of VEGF; that thalidomide only partially or temporarily blocks the production of this angiogenic protein; or that thalidomide does not significantly decrease VEGF within the neoplastic cells. Determining how thalidomide reduces the growth of metastasis is important because if a HSA exposed to thalidomide utilises other angiogenic pathways to sustain its progression, this would support the use of combination treatment with drugs that target these other pathways. Alternatively, if thalidomide results in only a minor or partial inhibition of

VEGF, then incorporation of other anti-VEGF drugs into the treatment protocol may help further decrease VEGF production, allowing greater inhibition of cancer growth and metastasis.

The goal of the current study was to assess whether levels of VEGF were lower in metastatic lesions of dogs treated with thalidomide compared with the metastatic lesions that occurred in dogs that did not receive thalidomide. The detection of reduced VEGF from metastatic tumours from treated dogs would support the hypothesis that thalidomide prolongs survival in dogs with HSA by inhibiting VEGF production by the neoplastic cells. Further, if VEGF was not detectible in the dogs with tumours treated with thalidomide, this would support the use of combination therapy to block other angiogenic pathways. Conversely, if VEGF is not reduced in the metastatic lesions or present at lower levels in the metastases from treated dogs this would support the use of other VEGF-blocking drugs as a way to improve the survival of dogs with HSA.

8.2 Materials and Method

8.2.1 Patients

Material was sourced from patients enrolled in the study reported in Chapter 7. Samples of metastatic lesions were collected by cosmetic necropsy. Only dogs that had no evidence of metastases by CT examination of the chest and abdomen were included in the study.

Sections of primary splenic HSA were also used from dogs for which metastatic lesions were available. This allowed comparison of VEGF immunostaining within the metastatic lesions after thalidomide to immunostaining within the primary lesions prior to thalidomide treatment.

A control population of metastatic HSA lesions was obtained from the pathology archive of the School of Veterinary Science, Massey University. None of these dogs had received thalidomide or any other anti-angiogenic or cytotoxic treatment for the management of their HSA. Tissues were obtained during post-mortem from dogs who had died or were euthanased after a diagnosis of splenic HSA.

8.2.2 Immunohistochemistry

Immunostaining for VEGF of the primary splenic HSA lesions in the treated dogs had been performed previously, as described in Chapter 7.

Tissue samples from the metastatic lesions from both the treatment and the control dogs had been stored as formalin-fixed paraffin embedded (FFPE) blocks. From these tissues, sections (5µm) were obtained from each individual specimen block and mounted onto positively charged glass slides. Each section was dewaxed in xylene and rehydrated in a graded alcohol series and equilibrated in phosphate buffered saline. VEGF immunostaining was performed as previously described in Chapter 4. Briefly, antigen retrieval was performed in a decloaker (Biocare Medical) at 100°C for 20 mins (VEGF) in a citrate buffer solution (EnVision™ FLEX Target Retrieval Solution (high pH), Dako Australia Pty. Ltd,). Immunostaining was then performed using a

Sequenza Immunostaining Center (Thermo Fisher Scientific). Endogenous peroxidase activity was blocked using a peroxidase-blocking reagent (EnVision™ FLEX, Dako Australia Pty. Ltd) for 15 mins. Tissue sections were incubated overnight with a 1:300 dilution of mouse antihuman VEGF polyclonal antibody [0.33µg /ml] (VEGF (A-20) sc-152: Santa Cruz Biotechnology Inc).[4-7] The presence of the antibody was detected using diaminobenzidine (DAB, Dako Australia Pty). For each batch of slides, positive and negative controls were used. Positive control tissues for VEGF were sections of a primary splenic canine HSA that had been used as the positive control in Chapter 3; this particular tissue sample was known to consistently stain avidly for VEGF. For negative control tissues, the primary antibody was omitted.

8.2.3 Evaluation of immunostaining

Each slide was assessed by light microscopy and immunostaining of VEGF was determined. The origin of the slide was not known to the evaluator during assessment of the immunostaining. Immunostaining was only evaluated in areas of well-preserved tissue morphology and away from areas of necrosis, tissue edges and other artefacts.

Immunostaining for VEGF was scored using a modification of a previously reported scheme.[8] With this system, tumours were scored based on the proportion of cells showing evidence of VEGF across 10 non-adjacent and non-overlapping fields using the following criteria: 1 (<50% cells weakly positive); 2 (<50% cells intensely staining); 3 (≥50% cells weakly positive); 4 (≥50% cells intensely staining). For dogs that had multiple metastatic lesions,

the VEGF scores for all the lesions was determined and the mean score used for that dog.

8.2.4 Statistical analysis

Statistical analysis was performed using SPSS (SPSS Statistics v26.0.0, IBM, New York, NY). The Mann-Whitney-Wilcoxon test was used to compare the immunostaining scores of the primary tumour with the metastatic lesions from the dogs treated with thalidomide, as well as the difference in the immunostaining scores of the metastatic lesions of the treated dogs with the control population. When multiple lesions for each dog were examined, the average score for all lesions was used for statistical comparison between treatment groups. $P < 0.05$ was considered significant.

8.3 Results

8.3.1 Thalidomide patients

Samples had been collected during a cosmetic necropsy examination from 5 of the 10 dogs that had been diagnosed with a stage II (no evidence of distant metastases) and had been treated with thalidomide as described in the previous study. Samples of metastases were collected from the liver from all 5 dogs. Metastases were also sampled from the mesentery in two dogs. Survival times after starting thalidomide treatment for these 5 dogs ranged from 157 to 653 days (mean 368 days).

8.3.2 Control dogs

Four dogs with metastases of a primary splenic HSA were available for this study. Metastases were collected from the liver and mesentery from three dogs while metastases were collected from liver, mesentery, and lung from the remaining dog.

8.3.3 Immunohistochemical scores

The immunohistochemical scores for each section of HSA are shown in Table 8.1. For the thalidomide treated group, immunostaining in the primary splenic HSA was widely distributed in all cases, with intense staining in 4 of 5 cases. In the metastatic lesions, immunostaining scores were between 1 and 2, with

Table 8.1:
VEGF immunostaining scores for treatment and control groups

Group	Case ID	VEGF score		Average score (metastasis)
		Primary lesion	Metastatic lesion	
Control	50174-a	-	4	3
	50174-b	-	2	
	49941-a	-	3	3
	49941-b	-	3	
	49941-c	-	3	
	40792-a	-	4	4
	40792-b	-	4	
	50542-a	-	4	4
	50542-b	-	4	
Thalidomide	64731-a	4	1	1
	P12016263-a	3	1	1
	P12016263-b	-	1	
	79220-a	4	1	1
	P14025177-a	4	2	2
	P14020625-a	4	2	2
	P14020625-b	-	2	

Figure 8.1:
Selected photomicrographs (400x) taken from four different metastatic lesions from the control group of dogs with haemangiosarcoma. The images show the high proportion of cells with intensely positive immunostaining (score 4) for VEGF in the dogs not receiving haemangiosarcoma.

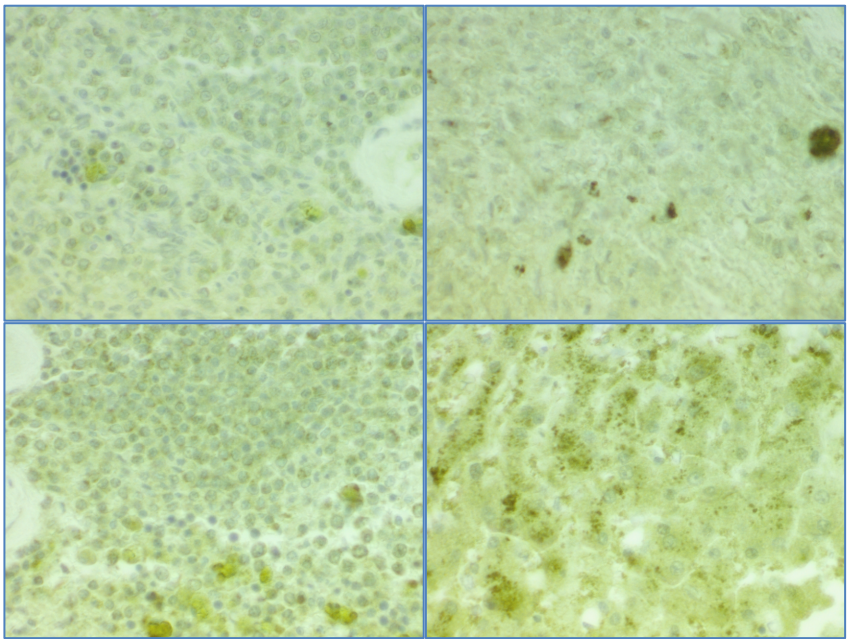
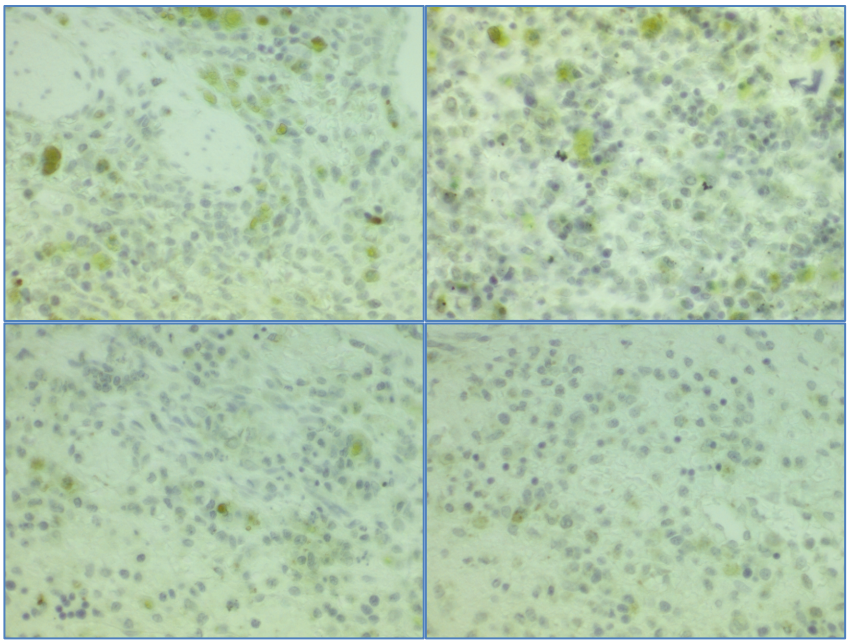


Figure 8.2:
Selected photomicrographs (400x) taken from four different metastatic lesions from the thalidomide treatment group of dogs with haemangiosarcoma. The metastatic lesions from dogs treated with thalidomide had <50% of cells with positive immunostaining for VEGF (i.e. score 1 or score 2).



less than 50% of cells showing evidence of VEGF protein within the cell (Figure 8.1).

For the control group, positive VEGF immunostaining was widely distributed across the tissues with 8 of 9 samples scoring a Grade of 3 or more (Figure 8.2).

The mean VEGF score within the metastatic lesions of the 5 dogs treated with thalidomide was 3.5 which was significantly higher than the mean VEGF score within the metastatic lesions of the 4 untreated dogs (1.4; p=0.02) The mean VEGF score within the metastatic lesions of the treated dogs was also lower than the mean VEGF score of the primary splenic lesions that were taken prior to the commencement of the thalidomide treatment (3,8; p = 0.02)

8.4 Discussion

Metastatic lesions from dogs treated with thalidomide had significantly lower VEGF immunostaining than both the original primary HSA tumour as well as metastatic lesions from dogs that did not receive this drug. These findings suggest that treatment with thalidomide reduces the production of VEGF by the tumour cell.

The ability for thalidomide treatment to reduce VEGF production within a cancer cell has been previously demonstrated in studies of human neoplasia. In an *in vitro* study using a cell-culture of human colon cancer cells, thalidomide treatment inhibited the expression of both VEGF-A and hypoxia-

inducible factor – 1α (HIF-1α).[9] Another study showed that serum VEGF was significantly reduced in multiple myeloma patients treated with thalidomide.[10] However, these studies have either relied on *in vitro* evidence of activity using cell cultures or by measuring the serum expression of VEGF. Only one previous study has directly evaluated the production of VEGF within neoplastic cells obtained from patients treated with thalidomide; this showed thalidomide reduced the immunostaining for VEGF by cells within prostate cancers.[11] The current study is, therefore, the first to demonstrate a positive impact of thalidomide treatment on VEGF production in a tumour from a non-human species. Additionally, this is the first time that VEGF production by a neoplastic cell within a sarcoma has been investigated and the first time that the effect of thalidomide on VEGF production in cells within metastatic lesions has been investigated in any species.

Because only a small number of cases were examined in the current study, it is possible the observed differences in VEGF immunostaining between the two populations are simply be due to natural variances in VEGF activity within HSA. This possibility is countered by the findings from previous studies that indicate the immunostaining of VEGF in both primary and metastatic canine HSA lesions is usually consistently strong.[12, 13] In one paper, which utilised a immunostaining scoring method similar to that used in the current study, more than 90% of tumours were scored grade 4, with the remaining tumours scoring between grades 2-3.[13] None of the tumours in that study were scored at 1 or less, which contrasts with the findings of the current study where metastatic HSA lesions in 3 of 4 (75%) dogs treated with

thalidomide had grade 1 immunostaining.[13] In another study, almost 90% of primary splenic HSA had more than 50% of cells with mild to strong immunostaining for VEGF; this would be equivalent to Grade 3 and 4 using the grading scheme of the current study.[14] This evidence would suggest that, even though there were only a limited number of metastatic HSA lesions examined from the thalidomide group, it would be unexpected for all of the examined tissues to have consistently low immunostaining for VEGF even if selection bias was a cause. This supports the hypothesis that thalidomide treatment actively reduces the production of VEGF protein within metastatic lesions.

Despite the significant reduction in VEGF in the tumours from dogs that received thalidomide, cancer progression was not completely halted with almost all dogs ultimately being euthanased due to their tumour. This inability to completely prevent cancer growth by inhibiting VEGF activity has been revealed by other studies. Although VEGF inhibitors have demonstrated clinical efficacy and improved survival in human patients with advanced cancer, most patients will eventually relapse.[15-18] Alternative pathways that enable angiogenesis to continue even when VEGF has been blocked have been described,[15] and include the platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and angiopoietin pathways. These VEGF-independent signalling pathways are able to stimulate endothelial cell migration and vasculogenesis by interaction with other receptors.[15, 18]

Because of these alternate pathways for angiogenesis, it is unfortunate the current study limited its focus to the effects of thalidomide on VEGF only, particularly as it is known that thalidomide has a range of other influences on other pathways within the tumour. As well as its potent effects on VEGF signalling, thalidomide is also known to inhibit the production of other angiogenic cytokines including FGF, HGF, interleukins 6 and 1β (IL-6 and IL-1β) and tumour necrosis factor-α (TNF-α), amongst others.[1] A more complete understanding of the effects of thalidomide on the metastatic lesion would have been possible if the presence or absence of these cytokines had also been examined. One of the observations at the start of this study was that if a HSA exposed to thalidomide is able to utilise other angiogenic pathways to sustain its progression, this would support the use of combination treatment with drugs that target these other pathways. Because the design of the current study was limited in its scope, no conclusion can be drawn on whether the tumour growth is simply slowed due to the reduced availability of VEGF, or whether other less efficient angiogenic mechanisms are brought into play.

A weakness of the current study is the limited number of cases examined. This limitation was mainly due to an inability to collect samples from dogs that died after being treated with thalidomide. These dogs typically died rapidly and unexpectedly due to spontaneous internal bleeding from a metastatic lesion. Although all owners had been asked to consent to a post-mortem of their dog, the reality was that due to the sudden and unexpected grief for the family, the requirement for post-mortem was either forgotten, or the veterinarian who performed the euthanasia was unaware of this need.

Furthermore, the candidate was often not informed of a dog's euthanasia until a day or two after the event, by which time the body had been sent for cremation. As a consequence of these failings, metastatic lesions were harvested from only half of the dogs that were diagnosed with stage 2 HSA prior to starting thalidomide treatment. The impact of this limited case material was minimised because there proved to be a sufficient difference between the immunostaining scores between the treated and control tissues to draw some conclusions about the possible effects of thalidomide on VEGF production. If there had not been such a magnitude of difference, it is likely that the small numbers of tissue samples available in the current study would have prevented any effect of treatment to be discovered.

Immunostaining of the primary and metastatic lesions was done using different methods. For the primary HSAs, VEGF immunostaining was done for diagnostic purposes at a commercial veterinary diagnostic laboratory. In contrast, the metastatic lesions were immunostained at Massey University. As the intensity of immunostaining contributed to the VEGF score, it is possible the metastatic lesions had lower scores because the immunostaining method at Massey University resulted in less intense immunostaining than at the commercial lab. However, the same antibody was used at Massey University and in the commercial lab suggesting a lower intensity was not due to lower affinity of the antibody to the VEGF in the metastatic lesions. Additionally, high intensity was seen in the metastatic lesions of the untreated dogs suggesting that the variance was due to the effects of treatment, rather than more extraneous influences.

As discussed in Chapter 5, the effects of fixation in formaldehyde and storage conditions of the FFPE block can have a variable effect on the integrity of proteins within the tissues, which may impact on the extent and intensity of immunostaining of cellular proteins.[19] However, for this study, tissues from dogs in both the control and treatment group likely experienced similar conditions from the time of tissue harvest at surgery or post mortem; in all cases, harvested tissue will have been immediately fixed in formaldehyde and transported to the laboratory for processing into paraffin blocks within 24 to 72 hours. This duration of fixation will have a minimal impact on antigen detection.[19] The quality of immunostaining is also considered quite stable for tissues that have been stored in FFPE blocks for very prolonged periods.[19] The one difference between the pre-analytical conditions for the tissues from the treatment and control group was the interval from death until tissues were harvested at post mortem. For dogs in the treated group, it is known that metastatic lesions were harvested almost immediately after euthanasia, or within 24 hours at most. However, for dogs in the control group it is plausible there will have been a delay of several days from the time of death until the dog underwent post mortem examination. The impact of post mortem delay on immunohistochemical staining has been studied in humans. In one study, no differences were observed in the immunohistochemical staining characteristics of several proteins in brain tissues despite post-mortem intervals of over 50 hours.[20] However, significant increases in VEGF gene expression has been reported in the blood of patients after a post mortem delay of over 12 hours.[21] However, no changes in VEGF gene expression were observed in the myocardium or

pericardial fluid at these same time points. Because VEGF production is primarily driven by increases in HIF,[22] it is plausible that profound hypoxia at the time of death could cause an acute drive for increased VEGF expression within cells in circulation. However, it is unlikely there could be any substantial alterations in protein production within other cells in the body after death. Based on this evidence, it can be presumed that the differences in VEGF immunostaining observed in the current study are not a result of variances in tissue handling and storage but do reveal the effects of thalidomide on VEGF protein production within the cells.

In hindsight, it would also have been useful to evaluate the presence of VEGF immunostaining in the metastatic lesions from dogs that were diagnosed with stage 3 HSA prior to starting thalidomide treatment. In the previous chapter it was suggested thalidomide treatment may make less of a difference in advanced disease because the angiogenic switch may become self-sustaining beyond a certain point in tumourigenesis. Anti-angiogenic treatments may therefore be less effective against macroscopic lesions that have an established tumour vasculature and microenvironment. The investigator chose not to include tissues from stage 3 dogs as there was only a limited number of tissue samples available. It was also impossible to know whether the metastatic lesions that had been randomly sampled at post-mortem had been present at the start of the study, or whether they had developed while thalidomide was being received. These factors would have influenced the interpretation of any findings in this small number of dogs. By comparison, dogs with stage 2 HSA should not have had any macroscopic metastatic lesions at the start of the study, so it could be presumed that any

of the gross tumours harvested at post-mortem would have achieved this growth despite continuous exposure to the anti-angiogenic effects of thalidomide.

8.5 Conclusion

This study provides evidence that the metastatic HSA lesions that developed while exposed to thalidomide have a significantly reduced VEGF immunostaining compared to both the original primary HSA and metastatic HSA lesions from dogs that have not been exposed to this drug. The findings of this study support continued investigation into how the diverse effects of thalidomide may prove effective in slowing the progression of HSA and other soft tissue sarcoma.

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Chapter 9

Conclusions and Future Directions

9.1 Introduction

The main aim of the studies contained in this thesis was to investigate previously unexamined aspects of soft tissue sarcoma (STS) biology to identify new prognostic markers for these common neoplasms. This was achieved by establishing a large archive of STS that had previously been resected in general practice. This tissue was then analysed using immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) techniques to understand the role of two molecules – vascular endothelial growth factor (VEGF) and decorin – in influencing tumour behaviour. This study revealed that when the tissue stroma surrounding the tumour cells had a strong immunostaining intensity for decorin, the risk of tumour-related death was significantly reduced. In addition, STS with a high immunostaining for VEGF were more than 7 times more likely to recur, and 5 times more likely to cause the death of the dog, compared to a STS with low immunostaining. When the immunostaining characteristics for VEGF and decorin were combined with other patient and tumour features into a predictive algorithm called a nomogram, it was possible to determine, with almost 100% accuracy, which dogs would remain disease-free 3 years after surgery. Remarkably, this prediction was obtained independently of any knowledge about the excisional

status of the tumour. This suggests that the presence or absence of these molecules in the tumour microenvironment may support the survival and progression of residual microscopic tumour cells that remain in the wound bed, or alternatively are surrogate indicators of other features of tumour biology that impact on recurrence or metastasis.

The importance of VEGF in the progression of tumour growth was subsequently demonstrated by treating dogs with haemangiosarcoma (HSA) – a mesenchymal tumour with many characteristics similar to STS – with thalidomide. Thalidomide is a potent antagonist of VEGF, but also has a number of other modulating influences on the tumour microenvironment.[1] Dogs treated with thalidomide survived significantly longer than dogs that did not receive this drug, suggesting that thalidomide can slow the ability for residual microscopic tumour cells to develop into a grossly visible, and life-threatening tumour. An analysis of metastatic lesions that developed in dogs treated with thalidomide revealed that immunostaining for VEGF was significantly reduced. This suggests that thalidomide may be a useful adjuvant therapy for dogs with STS that are considered to be at high risk of recurrence after surgery, as determined by their VEGF immunostaining intensity.

9.2 Importance of prognostic markers in soft tissue sarcoma

Surgery is generally accepted to be the best treatment for a STS. Because local recurrence was commonly observed in early studies of this tumour,[2] it has been traditionally argued that wide resection of normal tissues about the

tumour would provide the best outcomes for affected patients.[3, 4] However, the results of the retrospective analysis of 350 dogs with STS performed in this study suggested that the extent of surgical margins is not influential on tumour recurrence or patient survival. This is consistent with the findings of other authors,[5-7] and suggests it is likely that some STS could be successfully managed with narrow margins. However, there is currently no way to determine which STS will be cured by narrow surgical margins; many of the existing prognostic indicators that have been described - such as size, tumour mobility and histological characteristics such as necrosis - lack sufficient distinguishing ability to be used to predict patient outcomes with any confidence.[8] These limitations were confirmed in the current study, where a nomogram based on clinical characteristics alone had a limited ability to identify dogs at risk of recurrence after surgery. Tumour grade remains the most validated criteria to be correlated with the extent of resection margin, but grade determination may be unreliable in almost 15% of cases.[9, 10] In this study, grade alone had a poor ability to predict outcome after surgery, with a 53% false-positive and 17% false-negative prediction of recurrence. If prognostic markers are to be used to help determine the need for adjuvant therapy after surgery, or even to provide a guide to the actual surgical margins used to remove the tumour safely, they need to be reliable and accurate to enable clinical decisions to be made with confidence.

The prognostic markers selected for evaluation in the current study were chosen based on the recognition that a microscopic tumour remnant must re-establish a vascular supply if it is to grow into a clinically detectable cancer.

Both VEGF and decorin have been previously identified as having a pivotal role in enabling or supporting the growth of a new blood supply in cancer, a process known as angiogenesis. Their role in the prognosis of STS has not previously been studied. In the study reported in this thesis, VEGF was shown to have an important influence on both recurrence and survival of the STS. Evidence of an association between tumour recurrence and the absence of decorin within the tissues was less strong, but this may have been due to small patient numbers. Because decorin is likely to have a protective influence on VEGF isoforms that are strongly matrix-bound by sequestering them from cellular interactions, it is unfortunate that efforts to analyse the proportions of different splice variants of VEGF within the STS failed. It would have been helpful to have this information to allow the relationship between VEGF, decorin and tumour progression to be better understood. Nevertheless, the results of the study support the use of VEGF or decorin as prognostic markers for STS. More work needs to be performed to validate the nomogram with another tumour population. Inclusion of information on the status of histological margins into the nomogram may also reduce the prevalence of false-positive predictions. Because decorin was significantly correlated with the histological grade of the tumour, immunostaining could also be used to help improve the reliability of this important prognostic indicator.

9.3 Importance of VEGF as a therapeutic target

Local recurrence of STS after surgery occurs in about 15% of patients.[8] Metastatic disease may also develop in up to 40% of dogs. Although

chemotherapy and radiotherapy have both been suggested to prevent or slow the development of either local or distant recurrence of the STS, the efficacy of these treatments is difficult to determine from the current literature. Alternative adjuvant treatments for STS are required.

Having established that VEGF tended to be high in tumours at risk of recurrence, the next step in this study was to use this prognostic marker as a therapeutic target. Thalidomide was identified as an ideal drug to target VEGF with the intent of slowing or preventing tumour regrowth both at the original wound site, but also for distant metastasis. In this study, the benefits of thalidomide were more obvious in dogs when any residual tumour was too small to be detected on CT scan. In these patients, progression of the recurrent tumour was significantly slowed, with treatment reducing the daily risk of dying from their HSA by 80%. For dogs with Stage 2 HSA receiving thalidomide, median survival times increased from 49 days to more than 300 days; almost a third of the treated dogs lived for more than one year after surgery. Benefits of thalidomide treatment were less evident in dogs with existing gross metastatic lesions. Macroscopic HSA lesions are fragile and prone to spontaneous bleeding. Because thalidomide is not cytotoxic, treatment will not make existing metastatic lesions reduce in size, so any benefits of treatment may be overshadowed by the increased risk of spontaneous, life-threatening haemorrhage in these patients. Examination of metastatic lesions in dogs treated with thalidomide revealed that immunostaining for VEGF was significantly reduced, but not completely inhibited. It is possible that combining thalidomide with directly cytotoxic

agents such as doxorubicin may result in a synergistic improvement in the outcome for dogs with HSA.

9.4 Study limitations

A major limitation of the studies described in this thesis was the inability to determine whether the tumour had actually been completely removed by surgery. This was because all of the necessary sections of the tumour that were required to analyse the margins histologically had not been made available when the archive of STS used for this study was created. While it is assumed that the surgeon operating on the STS removed all visible traces of the tumour, demonstration of a resection margin that was free of tumour cells on microscopic examination would have provided more confidence that the prognostic markers identified in this study had an independent influence on the tumour. While the status of the histologic tumour free margin (HFTM) does not necessarily provide a consistent prediction for whether tumour recurrence will or will not occur, there would be additional information provided by this examination that could have greatly improved the conclusions of this study. Previous work in human STS has demonstrated the prognostic significance of a tumour profile that is either expansile or infiltrative,[11] and also the distribution of satellite lesions that radiate from the tumour pseudocapsule. If the tissue sections of STS used in this study could have been examined for these characteristics, it is possible the nomogram that was developed in this study may have been more accurate at predicting patients where tumour recurrence was more likely after surgery. Furthermore, correlating the expression of VEGF and decorin with different

tumour profiles may also have given more insight into the role of the biomolecules in the tumour microenvironment.

Several other limitations of this study have been discussed in previous chapters. It is important to reiterate that the clinical data for the STS used to establish the prognostic discoveries in this study was derived retrospectively. The tissues used for IHC and RT-PCR had also been fixed in formalin and stored in less than ideal conditions for more than 10 years before being studied. These factors introduce the potential for bias and inaccuracy, which could mean the conclusions of this study may not be valid. It would be important to verify the findings of this study with clinical data that has been collected prospectively, and to repeat the biomarker assays using tissues that have been recently collected to minimise the potential for artefactual distortion of the findings.

In hindsight, it would also have been desirable if a wider panel of immunochemical markers was applied, in addition to VEGF and decorin. This would have allowed a better analysis of the role the tumour microenvironment may have in supporting tumour progression, particularly in relation to the formation of the pseudocapsule and the variance in tumour cell migration into the surrounding tissues. Some examples of additional molecules that could have been studied include: 1) lysyl oxidase (LOX), which has been correlated with variations in collagen cross-linkage and tumour mobility;[12] and 2) matrix metallopeptidase 9 (MMP-9) and tenascin-C, both of which have been shown to play a pivotal role in remodelling of the ECM in other cancers.[13] Additional immunostaining for transforming

growth factor- β (TGF-β), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and $CD4^+Foxp3^+$ regulatory T cells (Treg) would also have improved the understanding of the role of thalidomide, by evaluating its immunomodulatory effects on the tumour. However, apart from Foxp3, many of the antibodies available against these proteins have not been studied in canine tissues, so their inclusion in the study would have required validation of the immunostaining protocols.

9.5 Future directions

The studies presented in this thesis provide important new insights into the biology of STS, and suggest a benefit of using VEGF and decorin to improve the prediction of outcome for a STS after surgery. Additionally, knowledge of these markers may allow them to be targeted for therapy. However, the evidence from these studies are not sufficiently robust to be used to influence clinical judgement and treatment strategies for STS. As discussed above, it will be important to verify the key findings of this study with another clinical population, and to validate the immunostaining characteristics for VEGF and decorin using fresh tissue. In addition, prospective validation of the nomogram with a new study population needs to be completed, ideally with inclusion of data on the status of the histologic margin. This would allow this device to become an important tool for a clinician, to help identify dogs that may be of risk of recurrence following surgery. These dogs could then undergo further surgery or adjuvant treatment such as chemotherapy or radiotherapy.

For the future, one of the most important pieces of information for the cancer surgeon is an accurate determination of the extent of tissue that must be removed about a mass to achieve effective local control. Most of the existing literature on prognostic markers, including the current studies, have focused on providing information for a surgery that has already been performed. Few, if any, of these publications have focussed on influencing or directing appropriate surgical dosing, or in providing the surgeon with objective information to determine the optimal surgical strategy for an individual patient. Cancer surgery would be improved if we had foresight about the biologic potential of the tumour being operated upon. This would allow the surgeon to more confidently titrate the resection margins about a tumour based on the actual characteristics of the tumour. The findings of the current study lend themselves to two additional areas of investigation that could help address this clinical need. Firstly, and as discussed in Chapter 6, development of a PCR assay using cellular material harvested from the tumour by fine needle aspiration would provide the surgeon with an ability to measure the VEGF and decorin expression of the tumour even before surgery was performed. Such techniques have been used in several human cancers, to provide information about the presence of gene mutations or other prognostic features that may impact on prognosis or treatment.[14, 15] If a similar technique could be developed for canine STS, this strategy could supplant the requirement for an incisional biopsy, thereby providing a cost-effective and less invasive means of obtaining relevant prognostic information about the tumour.

The second strategy to provide pre-operative prognostic information about a STS, that is currently being investigated, would use whole genome sequencing techniques to identify the gene signature that correlates with various histological features that are likely to have a prognostic influence on STS. Similar strategies have been undertaken for several other canine cancers, including mast cell tumour,[16] histiocytic sarcoma,[17] and melanoma.[18] The phenotypic traits that would be studied for STS would include: 1) histological characteristics such as grade and necrosis, 2) a tumour profile that is either expansile or infiltrative; 3) the presence or absence of satellite tumour cells beyond the pseudocapsule; 4) increased angiogenic potential, based on increased VEGF and/or low decorin levels; and 5) tumour mobility. By correlating the genetic fingerprint of the tumour with these phenotypic attributes and outcomes for the dog, it is hoped to identify genetic markers that are associated with outcome (e.g. recurrence after surgery, metastasis and tumour-related death) or provide targets for novel treatment options.[19] These technologies can provide detailed, genome-wide molecular characterisation, and document thousands of individual DNA mutations and other genomic alterations.[20] Currently, it is planned to perform a comprehensive transcriptome analysis on a large tissue archive of almost 200 STS where the key phenotypic characteristics have previously been documented.

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Appendices

The Appendix contains copies of documents relevant to the studies contained in this thesis. This includes:

A1 Copy of soft tissue sarcoma Questionnaire

A2 Chapter 2 DRC 16-V3: Soft tissue sarcoma in the dog: part 1: A current review

A3 Chapter 2 DRC 16-V3: Soft tissue sarcoma in the dog: part 2: Surgical margins, controversies and a comparative review

A4 Chapter 3 DRC 16-V3: Canine soft tissue sarcoma managed in first opinion practice: Outcome in 350 cases

A5 Chapter 7 DRC 16-V3: Does thalidomide prolong survival in dogs with splenic haemangiosarcoma?

Vet Clinic Reference: [redacted] (Lab reference: «LabID»)

The name of the client is: [redacted]
The animal we are interested in is: [redacted], 9.0 yr old M GSD

The mass was diagnosed as: SPINDLE CELL SARCOMA
The mass was reportedly located on the: VENTRAL ASPECT OF NECK
The mass was received by the lab on: 12/20/2003

Tumour description

Location of tumour

- ☐ Limb
- ☐ Trunk
- ☐ Head
- ☐ Unknown
- ☐ Other…….....

NOTE: In some animals, more than one mass will have been removed at the time of the original surgery. Please record answers for the mass diagnosed as a soft tissue sarcoma only (e.g. haemangiopericytoma, fibrosarcoma, liposarcoma, spindle cell sarcoma etc).

Size

- ☐ <1cm
- ☐ 1-5cm
- ☐ >5cm
- ☐ Unknown

If the size of the mass was recorded or described in the clinical notes, please also write this below:

Palpable characteristic

- ☐ Discrete/relatively mobile
- ☐ Firmly attached to surrounding tissues
- ☐ Unknown

Pre-operative diagnostic tests performed

- ☐ Fine Needle Aspirate
- ☐ Chest X-ray
- ☐ Abdominal Ultrasound
- ☐ Other: .....
- ☐ Lymph Node Biopsy
- ☐ No tests performed
- ☐ Unknown

If performed, what where the results of the above tests?

_____

_____

_____

_____

_____

Degree of resection performed

- ☐ Incisional biopsy
- ☐ Marginal (i.e. immediately about the tumour)
- ☐ <3cm (i.e. a small margin of normal tissue)
- ☐ 3cm or more
- ☐ Amputation
- ☐ Unknown

Please fax completed questionnaires to: 0845 643 5131

Vet Clinic Reference: [redacted] (Lab reference: «LabID»)

Patient Name: CHARLIE, 9.0 yr old M GSD
Client Surname: KINGSLEY-TEMPLAR

Follow up	
Did local recurrence of the tumour occur following resection?	☐ Yes ☐ No ☐ Unknown *If yes when was recurrence noted?* Date:..... *Was histopathology or cytology performed to confirm the nature of the recurrence?* ☐ Yes ☐ No ☐ Unknown *How was the recurrence managed?* ☐ No further treatment ☐ Repeated surgery ☐ Unknown
Did distant metastasis of the tumour occur?	☐ Yes ☐ No ☐ Unknown *If yes, when was metastasis first noted?* Date:..... *Location of metastasis (if known):* *Was histopathology or cytology performed to confirm the nature of the metastasis?* ☐ Yes ☐ No ☐ Unknown *Were radiographs or other imaging studies performed to confirm the presence of metastasis?* ☐ Yes ☐ No ☐ Unknown
Is the animal still alive?	☐ Yes ☐ No ☐ Unknown *If yes or unknown, what date did you last see the animal?* Date:..... *If no, was the animal euthanased?* ☐ Yes ☐ No ☐ Unknown What was the date of death?..... *In your opinion was death/euthanasia related to the sarcoma?* ☐ Yes ☐ No ☐ Unknown

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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:	Jonathan Bray
Name/title of Primary Supervisor:	John Munday
Name of Research Output and full reference:	
Bray, J.P. Soft tissue sarcoma in the dog: part 1: a current review Journal of Small Animal Practice Vol. 57, Issue 10, pages 510-519	
In which Chapter is the Manuscript /Published work:	2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	100%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
All aspects of this literature review	
For manuscripts intended for publication please indicate target journal:	
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Date:	3.3.2020
Primary Supervisor's Signature:	
Date:	4.3.2020

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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*.

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Name of Research Output and full reference:	
Bray, J.P. Soft tissue sarcoma in the dog: Part 2: surgical margins, controversies and a comparative review Journal of Small Animal Practice Vol. 58, Issue 2, pages 63-72	
In which Chapter is the Manuscript /Published work:	2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	100%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
All aspects of this literature review	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	
Date:	4.3.2020
Primary Supervisor's Signature:	
Date:	4.3.2020

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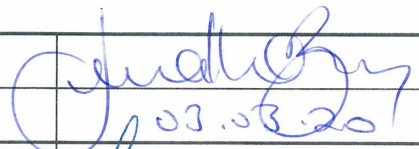
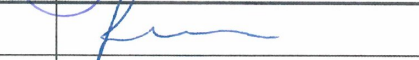
STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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:	Jonathan Bray
Name/title of Primary Supervisor:	John Munday
Name of Research Output and full reference:	
Bray, J.P., Polton, G.A., Mcsporran, K.D., Bridges, J., Whitbread, T.M. Canine soft tissue sarcoma managed in first opinion practice: Outcome in 350 cases	
In which Chapter is the Manuscript /Published work:	3
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	90%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Histology review (McSporran); Statistics (Bridges). Whitbread provided the tissue blocks and patient details for the study Polton is named as the original proposal/IP was from shared discussions.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	
Date:	03.03.20
Primary Supervisor's Signature:	
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Name of candidate:	Jonathan Bray
Name/title of Primary Supervisor:	John Munday
Name of Research Output and full reference:	
Bray, J.P., Orbell, G., Cave, N., Munday, J.S. Does thalidomide prolong survival in d Journal of Small Animal Practice Vol. 59, Issue 2, pages 85-91	
In which Chapter is the Manuscript /Published work:	7
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	95%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
All aspects of the study apart from: synthesis of thalidomide; collection of CT images; histologic and immumohistochemical analysis of tissue sections	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	
Date:	03.08.2020
Primary Supervisor's Signature:	
Date:	6 March 2020

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