Angiogenesis and Lymphangiogenesis in Canine Mammary Tumours

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‘Tutte le verità sono facili da capire una volta che sono state rivelate. Il difficile è scoprirle’

All truths are easy to understand once they are discovered, the point is to discover them

Galileo Galilei

Faculty of Pharmaceutical, Biomedical and Veterinary Sciences

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Angiogenesis and Lymphangiogenesis in Canine Mammary Tumours

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List of Abbreviations

Ang	angiopoietins
BEC	blood endothelial cell
BECP	blood endothelial cell proliferation
BV	blood vessel
BVA	blood vessel area
BVD	blood vessel density
BVP	blood vessel perimeter
CA9	carbonic anhydrase 9
CD31	platelet endothelial cell adhesion molecule-1, PECAM-1
CD34	hematopoietic progenitor cell antigen 34
CMT	canine mammary tumour
COP	comparative oncology program
Cox	cyclooxygenase
DAB	diaminobenzidine
DFS	disease free survival
EC	endothelial cell
ECM	extracellular matrix
ER	oestrogen receptor
FF	fibrotic focus
FGF	fibroblast growth factor
HE	haematoxylin-eosin
HGF	hepatocyte growth factor
Hif	hypoxia-inducible factor
IGF	insulin-like growth factor
IHC	immunohistochemistry
IMC	inflammatory mammary carcinoma
IT	intratumoural
LEC	lymphatic endothelial cell
LECP	lymphatic endothelial cell proliferation
LN	lymph node

LV	lymphatic vessel
LVA	lymphatic vessel area
LVD	lymphatic vessel density
LVP	lymphatic vessel perimeter
Lyve-1	lymphatic vessel endothelial hyaluronan receptor 1
NP	neuropilin
OS	overall survival
PDGF	platelet derived growth factor
PlGF	placental growth factor
PR	progesterone receptor
Prox-1	homeodomain protein prospero-related homeobox 1
PT	peritumoural
SLN	sentinel lymph node
TCP	tumour cell proliferation
TGF	transforming growth factor
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPF	vascular permeability factor
vWf	von Willebrand factor

CHAPTER 1: Current status of canine mammary tumour research, introduction to the tumour vasculature and comparative oncology

Partly based on

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1.1. General Introduction

Mammary tumours remain the most important cancer in women and bitches. In human breast cancer, angiogenesis and lymphangiogenesis in and around the tumour have been proven to be important for the tumour's growth and metastasis (Weidner *et al.*, 1991; Uzzan *et al.*, 2004; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009). As angiogenesis and in particular lymphangiogenesis research is limited in dogs, the presence and role of angiogenesis and lymphangiognesis in canine mammary tumours (CMTs) needs further investigation. Although much progress has been made in human breast cancer research during the last decades, the search for new and better therapeutics (e.g. directed to these vessels) with a minimum of adverse side effects remains necessary. Comparative oncology studies with dogs with CMTs can be useful to accelerate the drug development.

The focus of this work was to investigate in detail the presence and prognostic significance of these tumour-induced blood and lymphatic vessels in CMTs in order to develop an easily accessible and low-cost method to assess blood and lymphatic vessel characteristics that might have a prognostic value in routine diagnostics. In addition, a comparison with the situation in human breast cancer was made to promote dogs with CMTs as comparative oncology models for anti-(lymph)angiogenic therapies.

1.2. Canine mammary tumours

1.2.1. Prevalence of CMTs

Canine mammary tumours are the most common neoplasms in intact female dogs. Several European studies showed an incidence rate of approximately 200⁄100.000 dogs⁄year (Dobson *et al.*, 2002; Merlo *et al.*, 2008; Vascellari *et al.*, 2009). One out of 4 non-ovariohysterectomised dogs older than 4 years is expected to develop mammary tumours. In general, more than 40% of all tumours in female dogs are CMTs (Dorn *et al.*, 1968). The incidence of CMTs is decreasing in certain regions, such as the United States and some countries in Western Europe, because of the common practice of performing ovari(ohyster)ectomy at an early age, varying from 8 weeks to 7–12 months of age (Moe, 2001; Egenvall *et al.*, 2005). In contrast, in regions such as Scandinavia and Spain, the incidence of CMTs is much higher as preventive neutering is not routinely performed (Moe, 2001; Perez Alenza *et al.*, 2001; Egenvall *et al.*, 2005).

The caudal abdominal and inguinal mammary glands are mostly affected. Fifty to almost 70% of dogs with CMTs have multiple tumours (Benjamin *et al.*, 1999; Misdorp, 2002; Sorenmo, 2003; Sorenmo *et al.*, 2009). In case of multiple CMTs, different tumour types may occur within one animal (Misdorp, 2002; Sorenmo, 2003; Sorenmo *et al.*, 2009). Fifty per cent of the CMTs are malignant (Hampe and Misdorp, 1974; MacEwen and Withrow, 1996). The majority of malignant CMTs in dogs are carcinomas, and <5% of all CMTs are sarcomas (MacEwen and Withrow, 1996). Recently, one study hypothesized that malignant tumours can develop within pre-existing benign tumours (Sorenmo *et al.*, 2009). This study also showed that dogs with malignant tumours were significantly older than dogs with benign tumours, and malignant tumours were significantly larger than benign tumours. Two-third of the examined dogs had multiple tumours, and evidence of histological progression towards malignancy was seen with increasing tumour size. These findings suggest that CMTs can progress from benign to malignant. In addition, several publications reveal that dogs with histopathological pre-malignant lesions (hyperplasia, atypical hyperplasia and carcinoma-in-situ) (Antuofermo *et al.*, 2007; Mouser *et al.*, 2010) or

dogs with a previous history of malignant CMTs (Stratmann *et al.*, 2008; Sorenmo *et al.*, 2009) show an increased risk of developing a new malignant CMT. This might be explained by the fact that the entire mammary tissue is under the same hormonal exposure. Consequently, multiple tumours may develop over time or may be present in various stages of transformation at the same time (Sorenmo *et al.*, 2009).

1.2.2. Risk factors of CMTs

Different factors have an influence on the development of CMTs. The most important ones are age, breed and genetic predisposition, hormones and growth factors, cyclooxygenase-2 expression, and diet (Schneider *et al.*, 1969; Schneider, 1970; Rutteman, 1990; Sonnenschein *et al.*, 1991; Perez Alenza *et al.*, 1998; Queiroga *et al.*, 2005; Chang *et al.*, 2009; Lavalle *et al.*, 2009; Rivera *et al.*, 2009).

The prevailing theory is that most human breast cancers and CMTs originate from specific pre-malignant and benign lesions present in the glands. Women and bitches with a preceding diagnosis of atypical hyperplasia or carcinoma-in-situ seem to have an increased risk for a new primary mammary cancer (Arpino *et al.*, 2005; Hartmann *et al.*, 2005; Antuofermo *et al.*, 2007; Stratmann *et al.*, 2008; Sorenmo *et al.*, 2009; Mouser *et al.*, 2010). The risk is the highest in the ipsilateral side, but is also increased in the contralateral side suggesting that these pre-malignant lesions are precursors as well as predictors of later breast cancer or CMT development (Allred *et al.*, 2001; Antuofermo *et al.*, 2007; Hartman *et al.*, 2007; Stratmann *et al.*, 2008; Sorenmo *et al.*, 2009; Mouser *et al.*, 2010).

Age

Canine mammary tumours mostly develop in middle aged and old bitches (Dorn *et al.*, 1968; Hellmen *et al.*, 1993; Chang *et al.*, 2005). The reported median age of occurrence ranges from 8 to 10 years (Schneider, 1970; Hellmen *et al.*, 1993; Chang *et al.*, 2005). A recent study found a difference in the onset of benign and malignant tumours. Dogs with benign tumours had a mean age of 8.5 years, where dogs with malignant tumours had a mean age of 9.5 (Sorenmo *et al.*, 2009). Malignant tumours are rarely seen before 5 years of age (Perez Alenza *et al.*, 2000).

Breed and genetic predisposition

In general, purebred dogs were found to be significantly overrepresented among cases of CMTs (Schneider, 1970; Dorn and Schneider, 1976; Jitpean *et al.*, 2012). Some breeds in particular, e.g. Poodles, English Springer Spaniels, Brittany Spaniels, Cocker Spaniels, German Shepherds, Maltese, Yorkshire Terriers, and Dachshunds, seem to have an increased risk of developing a CMT. These observations suggest a genetic component (MacEwen and Withrow, 1996; Bronden *et al.*, 2003; Borge *et al.*, 2011). Nevertheless, a common genetic mutation has not been identified in dogs with CMTs.

Hormones and growth factors

The mammary tumours of both dogs and humans are hormonally controlled, and similarities in hormone dependency can be found between human breast cancer and CMTs (Thuroczy *et al.*, 2007). In women as in bitches, ovarian steroids stimulate the growth of normal mammary tissue under physiological conditions. Their proliferative effect on the epithelium may create conditions for neoplastic proliferation (Thomas, 1984; Genuth, 1998; Sorenmo *et al.*, 2000; Queiroga *et al.*, 2005). This occurs at every oestral cycle and makes the bitch more and more susceptible for further carcinogenesis (Rutteman, 1990; Stovring *et al.*, 1997; Chang *et al.*, 2009). Oestrogens promote ductal growth, whereas progestins are able to induce a lobulo-alveolar development of the mammary glands with hyperplasia of secretory and myoepithelial cells (Rutteman, 1990). During the long luteal phase of the canine oestrous cycle, the mammary gland is exposed to a high concentration of progesterone (Schaefers-Okkens *et al.*, 2005). The effect of ovarian steroids in bitches is mainly mediated by receptors expressed in the mammary tissue. Progesterone and oestrogen receptors (PR and ER, respectively) are present in both normal and neoplastic tissue. A decreased expression of ER and PR in CMTs seems to be correlated with a worse prognosis (MacEwen *et al.*, 1982; Rutteman *et al.*, 1988; Geraldes *et al.*, 2000; Nieto *et al.*, 2000; de Las Mulas *et al.*, 2005; Millanta *et al.*, 2005; Yang *et al.*, 2006; Chang *et al.*, 2009). In human breast cancer, similar results were reported (Pichon *et al.*, 1996; Bardou *et al.*, 2003).

The effect of pseudopregnancy on the development of CMTs is still debated, although, most likely, no effect is present (Rutteman, 1990; Gobello, 2001; Veronesi *et al.*, 2003; Schaefers-Okkens *et al.*, 2005).

Cyclooxygenase-2 (Cox-2)

Cyclooxygenases, particularly Cox-2, are involved in tumour development and progression in human breast cancer (Costa *et al.*, 2002; Denkert *et al.*, 2004). Recent studies have shown that Cox-1 and Cox-2 are expressed in canine CMTs (Dore *et al.*, 2003; Heller *et al.*, 2005; Millanta *et al.*, 2006b; Queiroga *et al.*, 2007; Dias Pereira *et al.*, 2009; Lavalle *et al.*, 2009; Queiroga *et al.*, 2011). Different studies provided evidence that Cox-2 expression is more frequent and more intense in malignant than in benign CMTs. Depending on the study, an over-expression of 56% to 100% of Cox-2 in tumour cells was found (Dore *et al.*, 2003; Heller *et al.*, 2005; Millanta *et al.*, 2006b; Queiroga *et al.*, 2007; Dias Pereira *et al.*, 2009; Queiroga *et al.*, 2010b). Following Lavalle et al. (2009), dogs with high Cox-2 expression in their CMT have a shorter survival time. Some authors did not find Cox-2 expression in normal mammary tissue (Dore *et al.*, 2003; Millanta *et al.*, 2006a; Millanta *et al.*, 2006b), whereas others did (Queiroga *et al.*, 2007). Recently, it has been shown that Cox-2 is also associated with angiogenesis and lymphangiogenesis (Queiroga *et al.*, 2011; Clemente *et al.*, 2013).

Diet

Several studies have evaluated the effect of various diet constituents and obesity on the risk of developing breast cancer in humans (Stoll, 2000; La Guardia and Giammanco, 2001; Cleary *et al.*, 2010) and CMTs in dogs (Shofer *et al.*, 1989; Sonnenschein *et al.*, 1991; Perez Alenza *et al.*, 1998). Obesity and a high fat diet have been linked to an increased risk of breast cancer in women. Obesity at one year of age or one year before the diagnosis of mammary nodules is a risk factor for developing mammary gland dysplasia or CMTs (Shofer *et al.*, 1989; Sonnenschein *et al.*, 1991; Perez Alenza *et al.*, 1998).

1.2.3. Classification of CMTs

Different classification methods of CMTs have been proposed, some of them were adapted from human classification systems (Misdorp *et al.*, 1972; Hampe and Misdorp, 1974; Misdorp *et al.*, 1999). The most recently published World Health Organization (WHO) International Histological Classification of Mammary Tumours of the Dog dates from 1999 and is now generally accepted (Misdorp *et al.*, 1999; Martins *et al.*, 2002; Sorenmo, 2003; Chang *et al.*, 2005; Ferreira *et al.*, 2009; Andrade *et al.*, 2010; Clemente *et al.*, 2010a; Clemente *et al.*, 2010b). The WHO combines histogenetic classification, descriptive morphology and prognostic elements in their classification (Misdorp *et al.*, 1999). A recent review compares the WHO classifications of 1974 with the modification of 1999 and additionally discusses new histological subtypes that have been described since the publication of the WHO classification of 1999 (Goldschmidt *et al.*, 2011). Moreover, a new histological classification is proposed based on the previous published ones.

Most CMTs are of epithelial origin (simple adenoma/simple carcinoma), some consist of both epithelial and myoepithelial tissue (complex adenoma/complex carcinoma), a few tumours are of mesenchymal origin (fibroadenoma/fibrosarcoma/ osteosarcoma/other sarcomas), and frequently a combination of epithelial and mesenchymal tissue (mixed benign tumours/carcinosarcoma) is seen (Hampe and Misdorp, 1974; MacEwen and Withrow, 1996; Misdorp *et al.*, 1999; Misdorp, 2002). The first WHO classification used the term ‘adenocarcinoma’ in tubular and papillary epithelial malignant tumours (Hampe and Misdorp, 1974). Misdorp et al. (1972) defined ‘adenocarcinoma’ as “a carcinoma of which the arrangement is predominantly glandular, with a lumen and which can be subdivided into tubular and papillary types”. The most recent WHO classification only uses the term ‘carcinoma’ for these malignancies (Misdorp *et al.*, 1999). In the different studies cited in this doctoral thesis, both terms have been used, depending on the classification method the concerning authors applied. In what follows, the terms as given in the original papers are used. The term inflammatory carcinoma is used for a clinical entity

characterised by a fulminant clinical course, sudden presentation with inflammatory characteristics of the mammary glands. Histologically, several types of highly malignant mammary carcinomas have been described and typically invasion of the superficial lymphatics by tumour cells can be recognised (Goldschmidt *et al.*, 2011). Malignant mesenchymal CMTs (sarcomas) are much less common than carcinomas. They comprise less than 5% of all CMTs, and less than 13% of all malignant CMTs. It is uncertain whether they arise from myoepithelial tissue that has undergone neoplastic change, from the intralobular connective tissue, or from pre-existing benign mixed tumours (Moulton *et al.*, 1970; Misdorp *et al.*, 1971; MacEwen and Withrow, 1996; Sorenmo, 2003). Different types can occur, with fibrosarcoma and osteosarcoma being the most common ones. Occasionally, chondrosarcomas and liposarcomas are found (Misdorp *et al.*, 1999).

Although the differentiation between benign and malignant tumours is based on various characteristics, it is not possible to clearly distinguish both types in all cases. It is estimated that misdiagnosis of malignant tumours as benign may occur in about 10% of the mammary tumours in the dog (Hampe and Misdorp, 1974; Misdorp, 2002). In general, benign tumours lack destructive or invasive growth and are often encapsulated. Necrosis, loss of differentiation, cellular and nuclear polymorphism, high mitotic index, high microvessel density and discontinuous basal membranes are typical features of malignant tumours (Mottolese *et al.*, 1994; Restucci *et al.*, 2000; Misdorp, 2002). Different inflammatory cells (lymphocytes, plasma cells, macrophages and mast cells) can be seen in CMTs. Their relation with malignancy is unclear (Misdorp, 2002; Estrela-Lima *et al.*, 2010; Woldemeskel and Rajeev, 2010; Raposo *et al.,* 2013)).

The different tumour types according to the WHO classification are presented in Table 1-1. A detailed description is beyond the scope of this doctoral thesis but can be found in the WHO guidelines (Misdorp *et al.*, 1999).

Table 1-1 *WHO classification of canine mammary tumours (adapted from Misdorp et al. 1999)*

Malignant Tumours	
Non infiltrating (in situ) carcinoma	An epithelial tumour with malignant features that has not invaded the basement membrane. Often multicentric.
Complex carcinoma	A tumour composed of both luminal epithelial and myoepithelial components.
Simple carcinoma	A carcinoma composed of one cell type, either resembling luminal epithelial cells or myoepithelial cells. Often infiltrative, lymphatic and/or hematogenous spread is common. Increasing malignancy: tubulopapillary → solid → anaplastic
• Tubulopapillary carcinoma	A tumour characterized by the formation of tubules and/or papillary projections.
• Solid carcinoma	A tumour characterized by the arrangement of tumour cells in solid sheets, cords, or nests.
• Anaplastic carcinoma	A highly infiltrative carcinoma of pleomorphic epithelial cells that is not classified in one of the other categories.
Special types of carcinomas	
• Spindle cell carcinoma	A malignant tumour composed of spindle cells that are usually arranged in epithelial patterns.
• Squamous cell carcinoma	A carcinoma composed of solid sheets and cords of cells with areas of squamous differentiation.
• Mucinous carcinoma	A carcinoma characterized by abundant mucin production.
• Lipid-rich carcinoma	A carcinoma characterized by cells with abundant vacuolated cytoplasm that contains a large amount of neutral lipid.
Sarcoma	
• Fibrosarcoma	A tumour of fibroblasts with various amounts of collagen
• Osteosarcoma	A sarcoma characterized by osteoid and/or bone formation by neoplastic cells
• Other sarcomas	Pure chondrosarcomas and liposarcomas are extremely rare.
Carcinosarcoma	A tumour composed of a carcinomatous and sarcomatous component.
Carcinoma or sarcoma in benign tumour	A tumour with foci or nodules of malignant appearing cells within a complex adenoma or benign mixed tumour.
Benign Tumours	
Adenoma	
• Simple adenoma	A tumour of well differentiated luminal epithelial or myoepithelial cells
• Complex adenoma	A tumour of luminal epithelial and myoepithelial cells
• Basaloid adenoma	A tumour of uniform cords and clusters of basaloid epithelial cells
Fibroadenoma	A tumour of a mixture of luminal epithelial cells and stromal cells, sometimes mixed with myoepithelial cells
• Low-cellularity fibroadenoma	
• High-cellularity fibroadenoma	
Benign mixed tumor	A tumour of benign cells resembling epithelial components (luminal and / or myoepithelial) and mesenchymal cells that have produced cartilage and / or bone and / or fat possible in combination with fibrous tissue
Duct papilloma	A branching or lobulated, simple or complex benign tumour in a distended duct.
Hyperplasia/Dysplasia	
Ductal hyperplasia	A non-neoplastic lesion characterized by an intraductal proliferation of epithelial cells, sometimes leading to partial or total obliteration of the lumen by epithelial hyperplasia
Lobular hyperplasia	
• Epithelial hyperplasia	A non-neoplastic proliferation of epithelial cells within intralobular ductules.
• Adenosis	A non-neoplastic proliferation of ductules.
Cysts	
Duct ectasia	A progressive dilatation of the mammary duct system.
Focal fibrosis (fibrosclerosis)	
Gynecomastia	Hyperplasia of ducts and stroma in male dogs.

Malignant epithelial CMTs (carcinomas) are graded histopathologically to provide important prognostic information to the clinician. Tumours with a higher grade have a worse prognosis (Elston and Ellis, 1991; Karayannopoulou *et al.*, 2005; Goldschmidt *et al.*, 2011). Different grading systems are used in veterinary medicine. Gilbertson et al. (1983) adapted a human classification system (Black *et al.*, 1975). Misdorp (2002) used a method similar to the Bloom and Richardson method, another human grading method. More recent work applies the Elston and Ellis method (also called Nottingham method), a modification of the Bloom and Richardson method, which is frequently used in human medicine (Elston and Ellis, 1991; Dutra *et al.*, 2004; Van der Auwera *et al.*, 2005; Gama *et al.*, 2008; Clemente *et al.*, 2010a; Clemente *et al.*, 2010b; Millanta *et al.*, 2010; Santos *et al.*, 2010). Gilbertson et al. (1983) based their classification method on numeric grading of 3 parameters: the degree of duct epithelial proliferation and of atypia in non-invasive neoplasms, the degree of nuclear differentiation in malignant proliferative lesions and the intensity of lymphoid cellular reactions. Both the Bloom and Richardson and the Elston and Ellis methods utilise similar criteria: tubule formation, nuclear pleomorphism and mitotic counts. The Elston and Ellis method (1991) is preferred since this method enables a more defined and more objective evaluation of the different criteria. Each of the criteria is scored on a scale from 1 to 3. The total score is then converted to the grade of malignancy: 3-5: grade I (well differentiated carcinoma), 6-7: grade II (moderately differentiated carcinoma), 8-9: grade III (poorly differentiated carcinoma).

1.2.4. Clinical signs and diagnosis of CMTs

Dogs with CMTs are presented at the veterinarian with one or more nodules within the mammary gland (Figure 1-1). Alternatively, the nodules can be an incidental finding at a routine control visit. Most of the dogs are clinically healthy when they are initially presented (Sorenmo, 2003). Dogs may show nonspecific symptoms such as fatigue, lethargy, weight loss, dyspnoea, cough, lymphedema or lameness if metastasis is widespread. The extent and location of the metastases determines the occurrence and severity of the clinical signs (MacEwen and Withrow, 1996; Misdorp,

2002; Sorenmo, 2003). Although carcinomas metastasise mainly via the lymphatics to the regional lymph nodes and the lungs, haematogenous metastasis may also occur (Misdorp and Hart, 1979; MacEwen and Withrow, 1996; Misdorp, 2002).

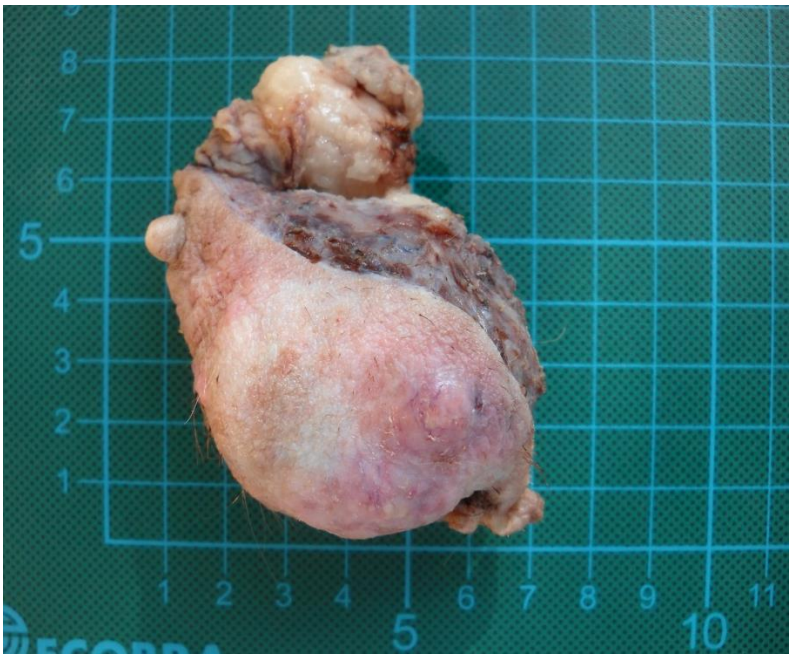


Figure 1-1 *Macroscopic image of a canine mammary tumour*

Physical examination comprises a general examination of the dog and a specific examination of the mammary glands. Ideally, the mammary glands are examined with the dog in dorsal recumbence. Every mammary gland needs to be thoroughly palpated and inspected. CMTs generally are firm, well demarcated nodules that vary in diameter from a few millimetres up to 10 to 20 cm. The overlying skin and the tumour itself may be traumatized and ulcerated (MacEwen and Withrow, 1996; Sorenmo, 2003). Palpation of the regional lymph nodes is mandatory. Normal axillary and superficial inguinal nodes are generally not palpable, while they can be felt if enlarged (Romagnoli, 2005).

In addition, cytological examination of fine needle aspirates (FNA) of CMTs can be performed. This is an easy and low cost procedure. Cytological criteria such as variable nuclear size, nuclear giant forms, high nucleus/cytoplasm ratio, variable nucleolar number, and abnormal nucleolar shape and size are significant predictors

for malignancy (Simeonov and Simeonova, 2006; Cassali *et al.*, 2007; Simon *et al.*, 2009). Cytological diagnosis can be hampered by the presence of a high number of non-tumour related or non-representative cells like stromal cells or inflammatory cells (Allen *et al.*, 1986; Martins *et al.*, 2002; Simon *et al.*, 2009). Therefore, if a tumour is suspected, resection and histopathological examination of the tumour is strongly recommended.

The sentinel lymph node (SLN) is the first lymph node to receive lymphatic drainage from a neoplasm. Biopsy of the SLN has become the standard of care in human oncology (Tuohy *et al.*, 2009). If positive, excision of the nodal basin is well advised. If negative, it is assumed that the rest of the regional nodes is negative too. Different authors describe the identification of SLNs in different canine tumours using FNA (Langenbach *et al.*, 2001), ultrasound (Nyman *et al.*, 2005; Nyman and O'Brien, 2007), micro-bubble contrast ultrasound (Lurie *et al.*, 2006; Gelb *et al.*, 2010), lymphoscintigraphy (Balogh *et al.*, 2002; Pereira *et al.*, 2008) or injection of dyes (Wells *et al.*, 2006). Lymph nodes should be aspirated or biopsied in every patient with CMTs as a part of the staging procedure (Tuohy *et al.*, 2009).

Medical imaging is mandatory (thorax radiographs in three directions and ultrasound of the abdomen) for evaluating the presence of distant metastases. Metastatic lesions in the thorax are usually circular well-defined radiodensities (Figure 1-2). However, they need to be at least 5 to 7 mm before they can be recognised on radiographs (Johnston, 1993; Kealy and McAllister, 2005; Nemanic *et al.*, 2006; Otoni *et al.*, 2010). Computed Tomography, which can detect lesions approaching 1 mm, depending on the scanner used, is therefore more sensitive than radiography (Nemanic *et al.*, 2006; Otoni *et al.*, 2010). Abdominal ultrasound can reveal abdominal metastases (Sorenmo, 2003).

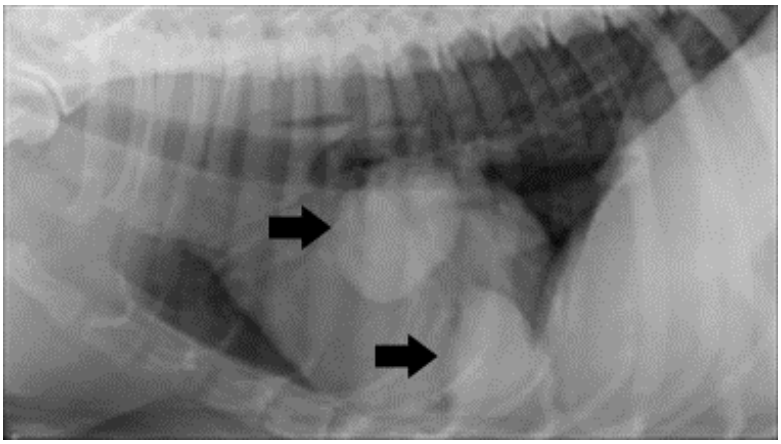


Figure 1-2 *Thorax radiograph of a dog with a malignant canine mammary tumour: thoracic metastases are indictated with arrows (Dierenartsencentrum Eversbos)*

Mammary tumours are staged according to the WHO TNM classification, which evaluates tumour size (T), lymph node involvement (N) and distant metastases (M) (Owen, 1980; MacEwen and Withrow, 1996). The original WHO staging system of Owen (1980) is more complex since the T and M categories have an additional subdivision taking into account fixation of the samples. MacEwen and Withrow (1996) simplified this original staging system, by not subdividing into different categories. Additionally, the modified system has 5 stages, where the original only has 4. In the original staging system, dogs with a small tumour (T1) and lymph node involvement are classified as stage II, as well as dogs with a larger tumour (T2) regardless of the lymph node status. Moreover, dogs with a large tumour (T3) will be stage III, regardless of the lymph node status. Dogs with distant metastasis are categorized as stage IV, independent of the tumour size or lymph node status. In contrast, according to the modified system, dogs without regional lymph node involvement and without distant metastasis are categorized in stages I, II and III, depending on their tumour size respectively. Each patient with regional lymph node involvement will be classified as stage IV and each patient with distant metastasis as stage V, regardless the tumour size (MacEwen and Withrow, 1996).

Definite diagnosis and grading of the tumour is based on histopathological examination as described above. If multiple masses are present, they should be evaluated individually, as different tumour types may occur within one patient (Misdorp, 2002; Sorenmo, 2003; Sorenmo *et al.*, 2009).

1.2.5. Treatment of CMTs

Surgery remains the gold standard treatment for most types of CMTs except for inoperable highly metastatic disease and for most of the inflammatory mammary carcinomas (MacEwen and Withrow, 1996; Misdorp, 2002). Surgical excision allows histopathological diagnosis and can be curative if the margins are devoid of tumoural cells and if the cancer has not spread (Fossum and Hedlund, 1997). Dogs with benign tumours and about 50% of dogs with malignant CMTs will be cured with surgery alone. The other 50% of the patients with malignant CMTs already has (micro)metastases at the time of surgery, which eventually will lead to death of the patient (Misdorp and Hart, 1979; Misdorp, 2002; Straw, 2005). The surgical approach depends on the tumour size, location and patient status. The different surgical techniques are: lumpectomy, simple mastectomy, regional mastectomy, unilateral mastectomy and bilateral mastectomy (Fossum and Hedlund, 1997). The different types of surgery, their indications and contraindications are presented in Table 1-2.

In unilateral and regional mastectomy involving the inguinal mammary gland, the superficial inguinal lymph nodes should be routinely removed. This is advisable because of the intimate anatomical association between the inguinal lymph nodes and the caudal mammary glands (Misdorp, 2002; Kirpensteijn, 2006). Although axillary lymph nodes resection is recommended when glands 1, 2 and 3 are affected, the resection is seldomly performed because of the difficult access to these nodes amongst others due to the proximity of the brachial plexus (Misdorp, 2002).

Table 1-2 *Surgical techniques for dogs with mammary tumors*

Type of surgery	Description	Indication	Contraindications
Lumpectomy/nodulectomy	Removal of small (≤ 5 mm), noninvasive tumor with an additional 1cm margin of normal appearing tissue	Small non-fixed nodules at the periphery of the gland	Multiple lesions Clinical signs of malignancy
Regional mastectomy	Resection of involved gland with glands associated with vascular and lymphatic drainage and with lymph nodes	Tumor in MG1 or MG2: resection thoracic and cranial abdominal glands Tumor in MG4 or MG5: resection of abdominal and inguinal glands Multiple tumors in adjacent glands Tumor in between MG1 and MG2 or MG4 and MG5	
Unilateral mastectomy	Removal of tumor with all ipsilateral glands	Multiple tumors throughout the mammary chain Tumor in MG3	
Bilateral mastectomy/ double chain resection	Removal of all mammary tissue	Multiple masses in different both mammary chains	Not recommended because of tension on the wound. Staged unilateral mastectomies are preferred with approximately weeks interval

MG = mammary gland

At the time of mastectomy, as in women, many dogs with malignant CMTs have established micrometastases, so despite surgery, the disease will progress (Gilbertson *et al.*, 1983). Although there are no established guidelines for treatment beyond surgery, dogs with locally advanced disease, with metastatic CMTs, or with a biologically aggressive histological type of CMT, may benefit from adjuvant treatment such as radiotherapy, anti-Cox-2 treatment, chemotherapy, desmopressin, hormonal therapy or anti-angiogenic therapy (Morris *et al.*, 1993; Lombardi *et al.*, 1999; Sorenmo, 2003; Simon *et al.*, 2006; Wedam *et al.*, 2006; Hermo *et al.*, 2008; Clemente *et al.*, 2009; Hunley *et al.*, 2010; Hermo *et al.*, 2011). The majority of these treatments is still in an experimental phase in veterinary medicine. Before a treatment is initiated, the risk versus gain balance should be strongly weighted, and the owner needs to be informed about the poor prognosis.

Recently, anti-angiogenic strategies have been developed in human breast cancer. Humanized monoclonal antibodies to vascular endothelial growth factor (VEGF) are one of these new therapies (e.g. bevacizumab). Those antibodies have an anti-angiogenic and thus an anti-tumoural effect in women with locally advanced breast cancer or inflammatory breast cancer (Ferrara *et al.*, 2005; Wedam *et al.*, 2006; Duda *et al.*, 2007; Burstein *et al.*, 2008). To the authors' knowledge, there is no information available on the use of anti-angiogenic therapy in CMTs and only a limited number of studies reported preliminary results of therapies with anti-angiogenic effect in other canine tumours (Burton *et al.*, 2011). However, most solid tumours, including CMTs, induce angiogenesis when growing. These numerous newly blood vessels provide the tumour cells with oxygen and nutrients and facilitate metastasis (Fox *et al.*, 1996; Restucci *et al.*, 2000). Malignant CMTs appear to have a higher microvessel density compared to benign ones (Restucci *et al.*, 2000; Restucci *et al.*, 2002; Restucci *et al.*, 2004). Therefore, at least theoretically, malignant CMTs in particular should be highly sensitive to anti-angiogenic therapy.

1.2.6. Prevention

Ovari(ohyster)ectomy at an early age can significantly reduce the risk of developing CMTs. Ideally, the procedure should be performed before the first oestrus, to maximize the benefits for CMTs, and after 3 months of age, to minimize the potential for developing urinary incontinence (Spain *et al.*, 2004; Root Kustritz, 2007). Bitches, which are spayed before the first oestrus have a 0.5% risk of developing mammary tumours compared to sexually intact ones; the ones who are spayed before the second oestrus have an 8% risk and those spayed after the second oestrus and before two and a half years of age have a 26% risk (Schneider *et al.*, 1969). However, a recent review questioned the protective effects of this early spaying (Beauvais *et al.*, 2012). As there are no significant differences between ovariectomy and ovariohysterectomy observed with respect to the incidence of long-term urogenital problems, including endometritis/pyometra, ovariectomy is the preferred method in the healthy bitch (Van Goethem *et al.*, 2006). However, as reproductive hormones

are involved in the pathogenesis of pyometra, it can be useful to make the abdominal approach for the gonadectomy wide enough so that the uterus can be evaluated. Another possibility is to perform an abdominal ultrasound prior to the surgery. If an abnormal uterus is seen, an ovariohysterectomy is mandatory (Ferguson, 1985; Sorenmo, 2003).

Literature shows contradicting results regarding the effect of ovari(ohyster)ectomy at a later age and at the time of CMT surgery (Brodey *et al.*, 1966; Schneider *et al.*, 1969; Misdorp, 1988; Sorenmo *et al.*, 2000). Older literature showed no prolonged survival of animals, which were spayed at the same time of the tumour surgery compared to animals that were left intact (Brodey *et al.*, 1966; Schneider *et al.*, 1969; Misdorp, 1988). These studies did not evaluate the oestrogen-receptor status of the CMT neither did they correlate the timing of ovario(hyster)ectomy in relation to the mastectomy with the survival times. In contrast, Sorenmo et al. (2000) reported that dogs, which underwent ovari(ohyster)ectomy concurrent with CMT surgery or less than 2 years before CMT surgery, significantly lived longer than dogs that were treated with tumour removal alone. It is hypothesized that the hormonal status of the dog influences the progression of CMTs.

1.2.7. Prognosis

The evaluation and comparison of the prognosis can be complicated because not all studies use the same endpoint. Prognosis is often expressed as the percentage of animals surviving 1 or 2 years after mastectomy (1- and 2- year survival) or as the overall survival (OS), which is the time from surgery to death (Hellmen *et al.*, 1993; Yamagami *et al.*, 1996; Philibert *et al.*, 2003; Chang *et al.*, 2005; Karayannopoulou *et al.*, 2005). However, survival time as an end point is often influenced by factors unrelated to CMTs. Therefore, some studies use the disease free survival (DFS) time, which is the time from surgical excision to the occurrence of metastasis or recurrences (Gilbertson *et al.*, 1983; Perez Alenza *et al.*, 1997; Pena *et al.*, 1998).

Survival can vary significantly depending on different tumour and host characteristics including age, tumour size, tumour stage, tumour histopathologic type, tumour grade, clinical behaviour of the tumour, lymph node involvement, expression of hormone receptors (ER and PR), Cox-2 expression, microvessel density, lymphoid cellular reactions in the tumour vicinity, cell proliferation markers and molecular genetic alterations. Ideally, different parameters need to be evaluated in order to obtain the best possible prognosis.

Increased ***age*** at diagnosis of CMTs has been associated with a shorter OS (Hellmen *et al.*, 1993) and a shorter DFS and OS (Perez Alenza *et al.*, 1997). Nevertheless, other investigators did not define age as a prognostic factor (Philibert *et al.*, 2003; Chang *et al.*, 2005). In analysing these results, it is important to bear in mind that older dogs are more likely to die from other, noncancerous, causes than younger dogs (Sorenmo, 2003).

Tumour size is one of the most important prognostic factors in CMT patients. A lower DFS or OS was reported for dogs with larger tumours (Yamagami *et al.*, 1996; Perez Alenza *et al.*, 1997; Philibert *et al.*, 2003; Ferreira *et al.*, 2009). Dogs with tumours larger than 3 cm in diameter showed a significant decreased OS, with a median of 14 months versus 22 months for dogs with tumours less than 3 cm in diameter (Philibert *et al.*, 2003), whereas Perez Alenza et al. (1997) found a low DFS and OS of 0 to 6 months with a larger tumour size. A tumour size of more than 3 cm was correlated with factors indicating poor prognosis such as loss of PR or higher proliferation index (Ferreira *et al.*, 2009; Sorenmo *et al.*, 2009). The likelihood of malignancy increased when the tumour reached a larger size (Sorenmo *et al.*, 2009). Lymph node metastasis was more likely in CMTs larger than 5 cm in diameter (Chang *et al.*, 2005).

Invasive growth combined with fixation to the skin and/or underlying tissue (which should be confirmed by histology) is a useful objective feature, often indicating a poor prognosis. Also, ulceration of the superimposed skin has been associated with malignancy (Hellmen *et al.*, 1993; Perez Alenza *et al.*, 1997).

Most of the malignant tumours eventually metastasise (Millanta *et al.*, 2005). ***Metastasis to the regional lymph nodes*** and ***distant metastasis*** are of prognostic relevance. A significant relationship between lymph node status and number of deaths was found (Yamagami *et al.*, 1996; Karayannopoulou *et al.*, 2005). Dogs with manifest metastasis at the time of diagnosis had a poorer prognosis (Yamagami *et al.*, 1996) with a median post-operative survival of 5 months compared to 28 months for dogs that lacked evidence of metastasis during diagnosis (Philibert *et al.*, 2003). When no metastases were detected at the time of diagnosis, metastases of epithelial CMTs generally occurred within a year in addition to an increased risk in higher histological grade CMTs (Sorenmo, 2003).

Dogs with a more advanced ***tumour stage*** have a significantly shorter survival expectation compared to dogs with a low stage disease (Yamagami *et al.*, 1996; Philibert *et al.*, 2003; Sorenmo, 2003; Chang *et al.*, 2005). Chang et al. (2005) found, after surgery, a median survival time of 6 months for dogs with stage IV and V. Most dogs with clinical stage I, II or III tumours were still alive 6 months after surgery. In addition, Philibert et al. (2003) showed that dogs classified as having stage I had the longest median post-operative survival of 24 months compared to 12, 15 and 19 months for stages II, III and IV respectively.

In the new WHO classification, the biological behaviour of the CMT corresponds with the ***histological differentiation***. Malignancy increases from non-infiltrating carcinoma over complex carcinoma over simple carcinoma tubulopapillary type over simple carcinoma solid type over simple anaplastic carcinoma to sarcoma (Misdorp *et al.*, 1999). Dogs diagnosed with the subtype anaplastic carcinoma had a significant shorter survival with a median postoperative survival of 2.5 months compared to 21, 16 and 14 months for adenocarcinoma (simple carcinoma subclass tubular carcinoma according to the most recent WHO classification (Misdorp *et al.*, 1999), solid carcinoma and the other histological subtypes respectively (Philibert *et al.*, 2003).

Histological grading of the canine mammary carcinoma is significantly related to prognosis: a high histological grade is associated with low OS and DFS (Perez Alenza *et al.*, 1997; Karayannopoulou *et al.*, 2005; Goldschmidt *et al.*, 2011; Pena *et al.*, 2013).

Lower expression of ***hormone receptors*** (ER and PR) is found in tumours with higher malignancy and worse prognosis (DFS and OS) (Nieto *et al.*, 2000; de Las Mulas *et al.*, 2005; Chang *et al.*, 2009). In addition, a higher percentage (85%) of dogs with malignant CMTs expressing ER and PR survived 1 year after surgery, compared to dogs with tumours expressing ER, but not PR (33.3%) (Chang *et al.*, 2009).

A statistical significant relationship between high ***Cox-2*** expression and poor survival was found in malignant CMTs (Lavalle *et al.*, 2009; Queiroga *et al.*, 2010a).

Increased ***microvessel*** density correlates with local recurrence, lymph node metastasis, and histological differentiation (Griffey *et al.*, 1998; Graham and Myers, 1999; Restucci *et al.*, 2000).

Lymphoid cellular reaction (lymphocytes and plasma cells) was assumed to indicate morphologic evidence of an anti-tumour immune response and has been associated with lower recurrence rates (Gilbertson *et al.*, 1983). Moreover, a recent publication demonstrated that the analysis of the inflammatory response in dogs with CMTs might provide important survival prognostic biomarkers. In contrast to the previous assumption, dogs with a high lymphocytic infiltrate showed lower OS (Estrela-Lima *et al.*, 2010). Further research is needed to clarify the importance of the infiltration of these cells in CMTs.

High expression of a cellular ***proliferation marker***, Ki67, is correlated with a higher risk for metastasis and death from neoplasia and with a low DFS and OS (Pena *et al.*, 1998; Ferreira *et al.*, 2009; Santos *et al.*, 2013).

Recently several ***molecular prognostic factors*** are being investigated. Literature shows contradicting results on the over expression of HER-2 (Human epidermal growth factor 2) in malignant CMTs (Ferreira *et al.*, 2009; Hsu *et al.*, 2009; Kim *et al.*, 2011). The prognostic role of BRCA-1 also still needs to be clarified (Nieto *et al.*, 2003; Rivera *et al.*, 2009). Three recent reports review the current knowledge on molecular markers for CMTs (Klopfleisch *et al.*, 2011; Rivera and von Euler, 2011; Sorenmo *et al.*, 2011).

1.2.8. A special type of CMT: Inflammatory mammary carcinoma (IMC)

Inflammatory mammary carcinoma is a special type of mammary carcinoma, for which limited information can be found on in literature. Although already 15 years old, the WHO-classification of 1999 is still the most recent and it does not describe IMC as a separate entity (Misdorp *et al.*, 1999). As discussed above, Goldschmidt *et al*. (2011) made a proposal for an adapted histological classification of CMTs, in which IMCs are described as a separate entity.

IMC is a rare, locally aggressive, rapidly growing and highly metastatic cancer that affects humans and dogs (Jaiyesimi *et al.*, 1992; Chang *et al.*, 1998; Kleer *et al.*, 2000; Perez Alenza *et al.*, 2001; Pena *et al.*, 2003). It represents a distinct clinical disease entity. Two clinical forms of IMC have been described in dogs (Perez Alenza *et al.*, 2001; Clemente, 2009). When an IMC develops in a patient with no history of mammary nodules, it is called primary IMC. This is the most aggressive form of IMC. Secondary IMC, by contrast, occurs in dogs with previous mammary tumours. Histologically, several types of highly malignant mammary carcinomas have been described. Most of the IMCs are anaplastic carcinomas (Sorenmo, 2003; de M. Souza *et al.*, 2009; Marconato *et al.*, 2009b, Goldschmidt *et al.*, 2011), although some studies describe other histological types such as tubular carcinoma, solid carcinoma, and malignant mixed tumours (Perez Alenza *et al.*, 2001; Pena *et al.*, 2003). The hallmark for the histological identification of IMCs is the invasion of dermal lymphatic

vessels by neoplastic cells. The blockage of these lymphatics by neoplastic emboli is responsible for the severe oedema in the region (Goldschmidt *et al*., 2011).

Typical clinical signs of IMC are a fulminant clinical course and sudden presentation of oedema, erythema, ulceration, pain, firmness and warmth of the mammary glands with or without mammary nodules. These signs of inflammation may mimic mastitis or severe dermatitis (Ginel *et al.*, 2000; Perez Alenza *et al.*, 2001; Pena *et al.*, 2003; de M. Souza *et al.*, 2009; Marconato *et al.*, 2009b; Clemente *et al.*, 2010b). Extensive lymphedema of the limbs may occur secondary to lymphatic infiltration or occlusion (Fossum and Hedlund, 1997). Metastases are usually already present at the time of diagnosis. Most studies reported mainly metastases in the regional lymph nodes and lungs (Perez Alenza *et al.*, 2001; de M. Souza *et al.*, 2009). However, a high incidence of metastases in the urinary bladder and in the reproductive tract (ovaries, uterine body and vagina) and a low incidence of metastases in the lungs, liver, kidneys or bone was found in one study (Clemente *et al.*, 2010b). Dogs with IMC have a mean survival time of 60 days after diagnosis (Marconato *et al.*, 2009b).

The percentage of VEGF and Cox-2 immunoreactive tumour cells is strikingly high (Millanta *et al.*, 2010). This finding may explain the high angiogenic phenotype of IMCs and may support their metastatic potential (Millanta *et al.*, 2010). Recent research showed that also lymphangiogenesis is induced in IMCs (Clemente *et al*., 2013).

Treatment of dogs with IMCs is mostly palliative. Because of the extremely invasive nature of IMCs, surgery is usually not recommended (Perez Alenza *et al.*, 2001; Clemente *et al.*, 2010b). Recent studies show that dogs with IMC may benefit from adjuvant treatment such as chemotherapy and Cox-2 inhibitors (Clemente, 2009; de M. Souza *et al.*, 2009).

1.3. Tumour vasculature

Solid tumours such as human and canine mammary tumours consist of tumour cells and tumour stroma, which differs from normal tissue stroma. Next to tumour vasculature, the stroma also contains extracellular matrix (ECM), (myo)fibroblasts, and a variety of inflammatory cells. This tumour microenvironment plays an important role in tumour initiation, maintenance, progression and metastasis. Moreover, continuous communication with the tumour parenchyma exists (Folkman, 1986; Ronnov-Jessen *et al.*, 1996; Elenbaas and Weinberg, 2001; Shekhar *et al.*, 2001; Pietras and Ostman, 2010; Holopainen *et al.*, 2011; Mao *et al.*, 2012; Watnick, 2012).

1.3.1. Blood vessels

Angiogenesis and alternative vascularisation mechanisms

When a solid tumour grows, it outgrows its oxygen and nutrient supply as the diffusion limit of oxygen is 100 to 200 µm. The resulting hypoxia is one of the most important trigger for changes and transformations to occur in the tumour stroma (Dor and Keshet, 1997; Zetter, 1998; Snyder *et al.*, 2008; Ferrara, 2009). One of the most important and most studied consequences of hypoxia is the induction of angiogenesis, the formation of new blood vessels out of existing ones, under influence of pro-angiogenic factors. (Folkman, 1986; Hanahan and Folkman, 1996; Dor and Keshet, 1997). Endothelial cells (EC) of quiescent blood vessels remain sensitive to angiogenic signals (Fagiani and Christofori, 2013). In adults, angiogenesis only occurs physiologically in the ovary, mammary gland and uterus during the normal female reproductive cycle and pregnancy as well as during wound healing (Dvorak, 1986; Augustin *et al.*, 1995; Carmeliet and Jain, 2000; Djonov *et al.*, 2001). These newly formed vessels rapidly mature and when the balance between pro- and anti-angiogenic factors is restored, the endothelial cells regain their quiescent state. In contrast, in various pathological conditions, including cancer, this balance is disturbed. Hypoxia in growing tumours will induce the production of pro-angiogenic factors by different cell types including neoplastic cells, endothelial cells, stromal cells, macrophages, plasma cells and lymphocytes. This causes an imbalance between

pro-angiogenic and anti-angiogenic factors, which results in an angiogenic switch (Forsythe *et al.*, 1996; Carmeliet and Jain, 2000; Carmeliet, 2005; Carmeliet and Jain, 2011; Fagiani and Christofori, 2013). Members of the vascular endothelial growth factor (VEGF) and angiopoietin (Ang) family are mainly endothelial specific and have a predominant role. Other growth factors, with a more general effect, are also involved in the angiogenic process, i.e. transforming growth factor α and β (TGF α and β), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) (Hillen and Griffioen, 2007; Karamysheva, 2008; Carmeliet and Jain, 2011)

The most important pro-angiogenic factor is Vascular Endothelial Growth Factor A (VEGFA), a member of the VEGF family, which also includes, VEGFB, VEGFC, VEGFD, VEGFE, VEGFF and placental growth factor (PlGF). These glycoproteins have specific transmembrane tyrosin kinase receptors, through which they exert their effect, i.e. VEGFR1 (FLT1), VEGFR2 (KDR, FLT2) and VEGFR3. Neuropilin 1 and 2 (NP1 and NP2) are also co-receptors (Tammela *et al.*, 2005a; Ferrara, 2009; Carmeliet and Jain, 2011). Increase of VEGFA generates endothelial cell proliferation, migration, survival and it enhances vessel permeability. Moreover, the initial name of VEGFA was vascular permeability factor (VPF). Paracrine VEGFA released by tumour, myeloid or other stromal cells increases vessel branching and gives rise to abnormal tumour vessels, whereas autocrine VEGFA released by endothelial cells, maintains vascular homeostasis (Lee *et al.*, 2007; Carmeliet and Jain, 2011). VEGFA mainly stimulates angiogenesis through binding with VEGFR2 (Carmeliet and Collen, 2000; Karamysheva, 2008; Lohela *et al.*, 2009). VEGFR2 is up-regulated in settings of physiological and pathological angiogenesis but down-regulated in endothelial cells of quiescent vessels (Lohela *et al.*, 2009). The exact role of VEGFR1 in angiogenesis is less clear. It may act as a decoy for VEGFA and in doing so, regulating the amount of free VEGFA available for the activation of VEGFR2 (Carmeliet and Collen, 2000; Ferrara, 2009; Carmeliet and Jain, 2011). VEGFA seems to be mainly regulating sprouting angiogenesis and less intussusceptive angiogenesis (Makanya *et al.*, 2005). Recent studies have shown that growth factors that were first considered to be lymphangiogenic, such as VEGFC and VEGFD and their receptor VEGFR3, can also

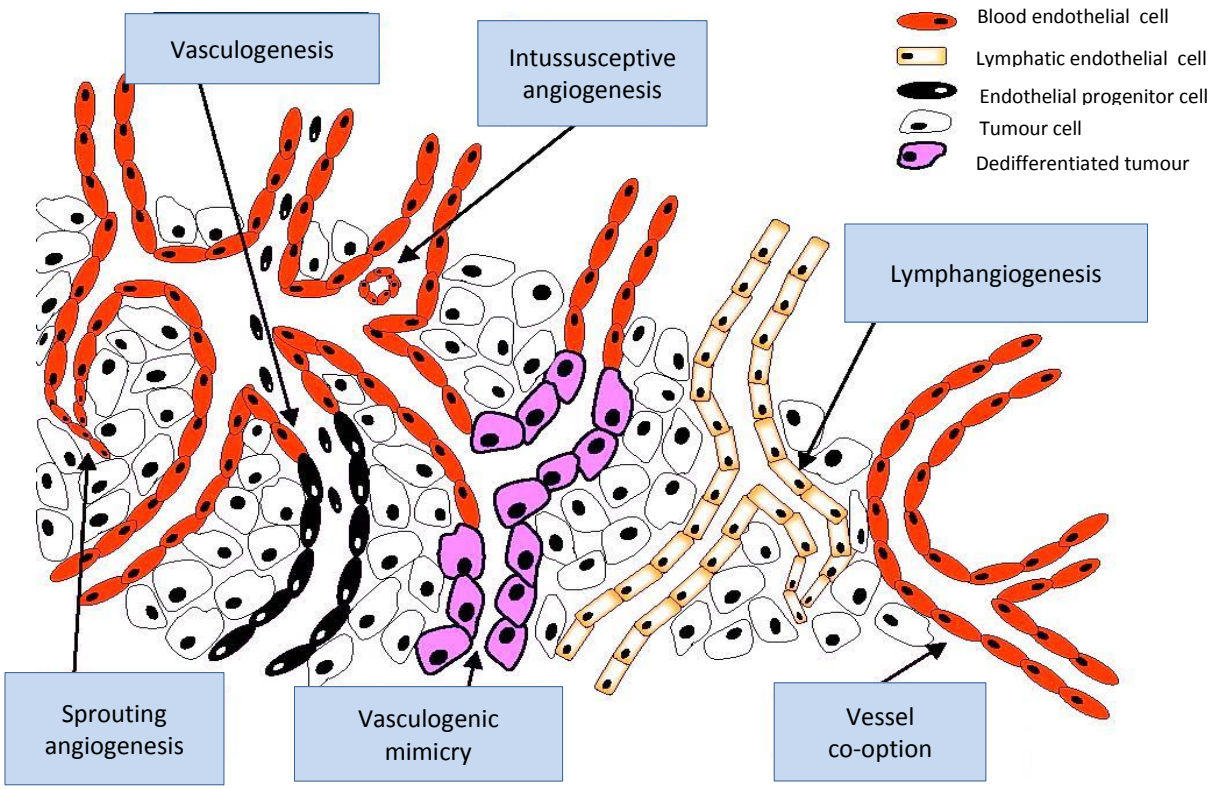
participate in angiogenesis (Scavelli *et al.*, 2004; Gomes *et al.*, 2013). In CMTs, both VEGFA and VEGFR2 have been investigated immunohistochemically (Restucci *et al.*, 2002; Restucci *et al.*, 2004; Millanta *et al.*, 2006c; Millanta *et al.*, 2010).

The most important partners of VEGFA in angiogenesis are the angiopoietins Ang1 and Ang2 and their receptor Tie2. The main effect of Ang1 is stabilisation of vessel walls, yet it can also mediate migration, adhesion and survival of endothelial cells and may counter the effect of VEGFA on permeability. In contrast, Ang2 disrupts initially the connections between the endothelium and perivascular cells and promotes cell death and vascular regression (Yancopoulos *et al.*, 2000; Fukuhara *et al.*, 2010; Fagiani and Christofori, 2013). Yet, in combination with VEGFA, Ang2 promotes neo-vascularization (Fagiani and Christofori, 2013). Angiopoietins seem to play an important role in intussusceptive angiogenesis (De Spiegelaere *et al.*, 2011; De Spiegelaere *et al.*, 2012). Ang1 and Ang2 have been analysed immunohistochemically in CMTs. Both have been identified in mammary carcinomas (Kato *et al.*, 2006; Klopfleisch *et al.*, 2011).

The last two decades, also in veterinary medicine, angiogenesis has become a subject of interest. Studies in canine tumours acknowledge large similarities in the angiogenic factors and pathways in human and canine tumours. VEGFA and his receptor VEGFR2 can be found in different canine tumours, including CMTs (Scheidegger *et al.*, 1999; Maiolino *et al.*, 2000; Restucci *et al.*, 2002; Rawlings *et al.*, 2003; Restucci *et al.*, 2004; Kato *et al.*, 2006; Al-Dissi *et al.*, 2007; Wolfesberger *et al.*, 2007; Qiu *et al.*, 2008; Al-Dissi *et al.*, 2009; Al-Dissi *et al.*, 2010; Queiroga *et al.*, 2011; Santos *et al.*, 2010; Wolfesberger *et al.*, 2012; Yhee *et al.*, 2012; Clemente *et al.*, 2013). In addition, Ang1 and Ang 2 were detected in different canine tumours (Kato *et al.*, 2006).

Most studies, when investigating angiogenesis in malignant tumours, only focus on sprouting angiogenesis, as it was long thought to be the only form of angiogenesis. However, another form of angiogenesis, the intussusceptive angiogenesis has been described. In addition, alternative vascularisation mechanisms may occur simultaneously with angiogenesis: vessel co-option, postnatal vasculogenesis and vasculogenic mimicry (Figure 1-3) (Carmeliet and Jain, 2000; Auguste *et al.*, 2005; Dome *et al.*, 2007; Hillen and Griffioen, 2007; Carmeliet and Jain, 2011; De Spiegelaere *et al.*, 2012).

Figure 1-3 *Different mechanisms of tumour vascularization. (Adapted from Hillen and Griffioen, 2007)*



Sprouting angiogenesis involves the formation of blood vessel branches as 'sprouts' of pre-existing vessels and is mainly regulated by hypoxia induced angiogenic growth factors. First, the VEGFA gradient selects a tip cell, which is located at the tip of the sprout and which defines the direction of the sprout with their numerous filopodia. Stalk cells are present just behind the tip cells and through their high proliferation rate the sprout elongates. The new sprout then fuses with an opposite sprout and a lumen will be formed. During maturation, stalk cells transform into the more quiescent phalanx cells (Dome *et al.*, 2007; Hillen and Griffioen, 2007; De Smet *et al.*, 2009; Carmeliet and Jain, 2011; Potente *et al.*, 2011).

In **intussusceptive angiogenesis**, connective tissue columns (intraluminal pillars) are inserted into the vessel lumen. These pillars subsequently grow and segregate the vessel lumen. Intussusceptive angiogenesis can only work on existing vessel networks formed through vasculogenesis or through sprouting angiogenesis. Intussusceptive angiogenesis can increase the complexity and density of the pre-existing microvascular network and in doing so playing a major role in growth and remodelling of tumoural vascular beds (Augustin, 2001; Djonov *et al.*, 2002; Kurz *et al.*, 2003; Auguste *et al.*, 2005; Dome *et al.*, 2007; Hillen and Griffioen, 2007; De Spiegelaere *et al.*, 2012).

Vessel co-option. During the initial phase of tumourigenesis, tumours can invade the host tissue and co-opt the pre-existing tissue vessels without effectuating angiogenesis. These vessels can even be completely integrated in the tumour. Generally, the co-opted vessels regress in a later stage as a result of host defence mechanism and other vascularisation methods will occur. Yet, it has been described that vessel co-option may persist during the entire period of primary and metastatic tumour growth (Holash *et al.*, 1999; Dome *et al.*, 2002; Dome *et al.*, 2007; Hillen and Griffioen, 2007).

Vasculogenesis, the formation of blood vessels from endothelial precursor cells, normally only occurs in the early (embryonic) vascular development. However, during tumour growth, the secreted pro-angiogenic factors may also mobilise bone marrow derived endothelial progenitor cells towards ischemic sites in the tumour (Asahara *et al.*, 1999; Moore *et al.*, 2001; Dome *et al.*, 2007).

Vasculogenic mimicry may occur in highly malignant tumours, e.g. melanomas and breast tumours. Genetically deregulated, aggressive tumour cells form a tubular microvessel-like network that connects to the adjacent circulation and enables blood flow throughout the tumour (Folberg *et al.*, 2000; Dome *et al.*, 2007; Hillen and Griffioen, 2007). Recent research in CMTs showed the presence of vascular mimicry in CMTs. In accordance to human breast cancer, inflammatory mammary cancers have a higher frequency of vasculogenic mimicry (Clemente *et al.*, 2010a).

As most anti-angiogenic therapies target sprouting angiogenesis, they are less effective when alternative vascularisation mechanisms are present. Moreover, in some cases where sprouting angiogenesis can be diminished by these therapies, a shift towards for example intussusceptive angiogenesis may occur (Hendrix *et al.*, 2003; Hlushchuk *et al.*, 2008). However, little is known on the relative implications of these alternative vascularisation methods in tumour development (Auguste *et al.*, 2005).

Tumour vessels are morphologically and functionally abnormal compared to normal blood vessels: tumour vasculature is highly disorganized and heterogeneous, vessels are tortuous with excessive and chaotic branching, dilated vessels with uneven vessel lumens can be found, as well as multiple bifurcations, loops and blind ending sprouts. This causes an heterogeneous blood flow with periods of intermittent flow, reverse flow, stasis and arterio-venous shunting, which distributes oxygen, nutrients, immune cells and drugs unevenly. Every layer of the vessel wall is abnormal. The endothelial cells are abnormal in shape and can grow on top of each other and project into the lumen, while pericytes and smooth muscle cells are lacking. Together with a discontinuous or absent basement membrane, this may cause numerous

fenestrae and widened interendothelial junctions, which makes tumoural vessels leaky. Despite the initial induction of angiogenesis, these changes create a hostile environment of hypoxia, acidosis and high interstitial fluid pressure, which in turn can induce more angiogenesis and select for more malignant cancer cells. In addition, the loosely assembled vessels facilitate tumour cell intravasation and dissemination (Fox *et al.*, 1996; Gulledge and Dewhirst, 1996; Carmeliet and Collen, 2000; Carmeliet and Jain, 2000; Carmeliet, 2005; Jain, 2005; Potente *et al.*, 2011)

1.3.2. Lymphatic vessels

Characteristics of the normal lymphatic vasculature

The lymphatic system is a one-way, open-ended complex network of capillaries, vessels, collecting vessels, lymph nodes, trunks and ducts that is essential for the maintenance of the fluid homeostasis in the body. It collects and returns lymph, composed of interstitial fluid components, metabolites and plasma proteins extravasated from blood capillaries, to the circulatory system. The lymphatic system also transports cells from the immune system and plays a role in the intestinal absorption of lipids (Swartz and Skobe, 2001; Alitalo *et al.*, 2005; Tammela *et al.*, 2005b; Thiele and Sleeman, 2006; Sleeman *et al.*, 2009; Schulte-Merker *et al.*, 2011).

The lymphatic capillaries are formed by a single thin, non-fenestrated, oak-leaf-shaped lymphatic endothelial cell layer, without the presence of pericytes and smooth muscle cells. The lymphatic endothelial cells have discontinuous button-like junctions that allow fluid and certain molecules and cells to enter into the vessels (Figure 1-4). The basement membrane is discontinuous or even absent in the lymphatic capillaries. Elastic fibres (anchor filaments) anchor the lymphatic endothelial cells to the extracellular matrix. They prevent the vessels from collapsing during changes in interstitial pressure and facilitate the uptake of soluble tissue components, even in high pressure environments. In contrast, the collecting lymphatic vessels have zipper-like interendothelial junctions, contain valves, which prevent lymph backflow, and have a coating of perivascular smooth muscle cells with

an intrinsic contractile capacity, allowing propulsion of lymph through the vessels (Leak, 1976; Witte *et al.*, 2001; Pepper and Skobe, 2003; Tammela *et al.*, 2005b; Cueni and Detmar, 2008; Alitalo, 2011; Schulte-Merker *et al.*, 2011).

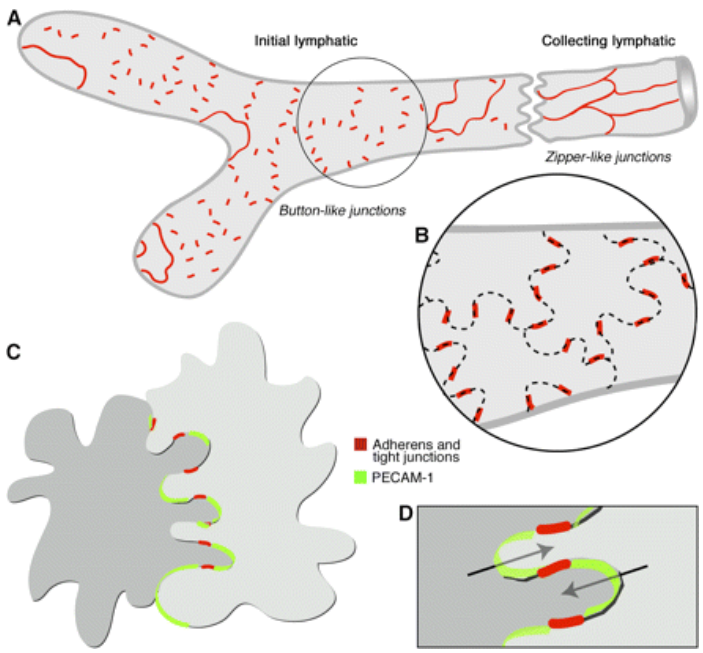


Figure 1-4 *Buttons in initial lymphatics border sites of fluid entry. (A) Schematic diagram showing distinctive, discontinuous buttons in endothelium of initial lymphatics and continuous zippers in collecting lymphatics. (B) More detailed view showing the oak leaf shape of endothelial cells (dashed lines) of initial lymphatics. Buttons (red) appear to be oriented perpendicular to the cell border but are in fact parallel to the sides of flaps. (C and D) Enlarged views of buttons show that flaps of adjacent oak leaf–shaped endothelial cells have complementary shapes with overlapping edges. Adherens junctions and tight junctions at the sides of flaps direct fluid entry (arrows) to the junction-free region at the tip without repetitive disruption and reformation of junctions (Baluk et al., 2007).*

Lymphatic drainage of the mammary gland

In general, the lymphatic system is important in the spread of tumour cells. Each mammary gland has a network of small lymphatic vessels which joins similar networks in the subcutis and conjugates in larger vessels running to the draining lymph nodes (Figure 1-5) (Miller *et al.*, 1964; Silver, 1966). Lymphatics of ipsilateral and contralateral glands can anastomose (Barone, 1990). Usually, the cranial and caudal thoracic glands drain to the axillary lymph centre (proper axillary lymph node and accessory axillary lymph node if present) on the ipsilateral side, and the caudal abdominal and inguinal glands drain to the ipsilateral inguinofemoral lymph centre (superficial inguinal lymph nodes, some authors call these mammary lymph nodes). Variations on the drainage pattern of the cranial thoracic glands and the caudal abdominal glands have been reported. The lymphatic drainage of the cranial abdominal glands conveys to in the axillary and inguinofemoral lymph centre (Barone, 1990; Patsikas and Dessiris, 1996a; Patsikas and Dessiris, 1996b; Pereira *et al.*, 2003; Patsikas *et al.*, 2006). Patsikas *et al*. (1996a,b) and Patsikas *et al*. (2006) did not find any lymphatic anastomoses in normal mammary glands between the different glands. Yet, they proved that lymph can pass by retrograde flow from one gland to another, through their common regional lymph node, and that there is a connection between the superficial inguinal lymph nodes from either side. Pereira et al. (2003) on the other hand reported anastomoses even in healthy mammary glands between lymph vessels of ipsilateral and contralateral glands. Neoplastic glands presented more anastomoses compared with healthy ones. It was stated that mammary neoplasia can change the lymphatic drainage pattern by forming new drainage channels (Pereira *et al.*, 2003).

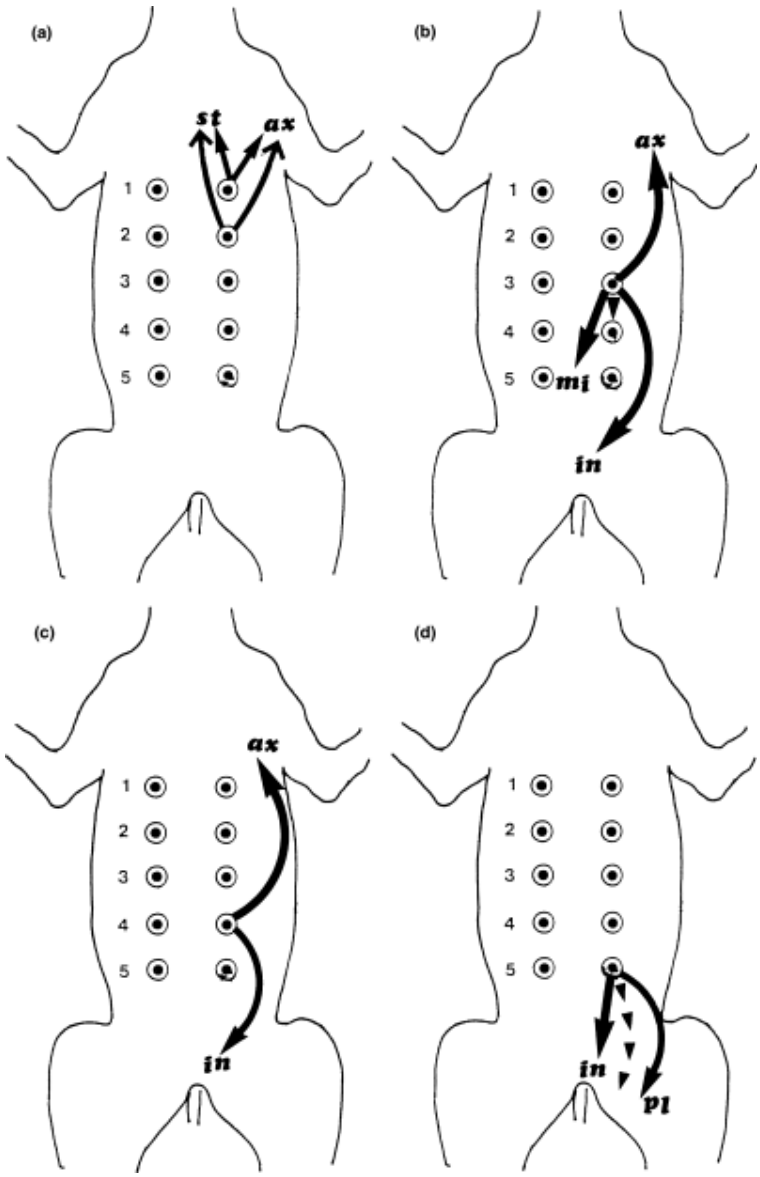


Figure 1-5 *Schematic representation of the lymph drainage from the first or cranial thoracic and second or caudal thoracic (a), third or cranial abdominal (b), fourth or caudal abdominal (c) and fifth or inguinal (d) neoplastic mammary glands in the bitch (1, first mammary gland; 2, second mammary gland; 3, third mammary gland; 4, fourth mammary gland; 5, fifth mammary gland; ax, axillary lymph nodes; st, sternal lymph nodes; mi, medial iliac lymph nodes; in, superficial inguinal lymph nodes; pl, popliteal lymph nodes) (Patsikas et al., 2006).*

Lymphangiogenesis

Lymphangiogenesis, the formation of new lymphatic vessels out of pre-existing ones, is closely related to angiogenesis. Lymphangiogenesis occurs in adults only during inflammation, wound healing, tissue regeneration and tumour growth (Reis-Filho and Schmitt, 2003; Al-Rawi *et al.*, 2005a; Thiele and Sleeman, 2006; Cueni and Detmar, 2008). Hypoxia and inflammation cause a shift towards pro-lymphangiogenesis, the lymphangiogenic switch, comparable to the angiogenic switch (Eccles *et al.*, 2007; Cao, 2008; Ran *et al.*, 2010). Tumour lymphatic vessels are mainly formed by direct vessel co-option, sprouting and/or splitting from pre-existing lymphatic vessels (He *et al.*, 2004). Also similar to angiogenesis, lymphangiogenesis is induced by a large number of lymphangiogenic factors produced by tumour cells, stromal cells, and inflammatory cells, with a key role for members of the VEGF family (in particular VEGFC and VEGFD) and their receptor VEGFR-3 (Al-Rawi *et al.*, 2005b; Cao, 2005; Karpanen and Makinen, 2006; Van der Auwera *et al.*, 2006; Cao and Zhong, 2007; Lohela *et al.*, 2009; Sleeman *et al.*, 2009; Holopainen *et al.*, 2011). VEGFC has also been reported to induce hyperplasia and dilation of the pre-existing and tumour lymphatic vessels and to increase lymph flow rate, which may have an impact on lymph node metastasis (Skobe *et al.*, 2001b; Stacker *et al.*, 2001; Cao and Zhong, 2007; Harrell *et al.*, 2007). Experimental blocking of the VEGFR-3 pathway, suppresses the formation of new lymphatic vessels (Ran *et al.*, 2010). Traditionally, VEGFC and VEGFD were considered to be only lymphangiogenic via activation of VEGFR-3, predominantly expressed on normal and malignant lymphatic endothelial cells and stromal cells (including macrophages) (Oh *et al.*, 1997; Achen *et al.*, 1998). However, extensive research in the field has showed that there is overlap between the angiogenic and lymphangiogenic pathway in both directions. A direct effect of VEGFA via VEGFR-2 on lymphatic endothelial cells has been shown (Cursiefen *et al.*, 2004; Hong *et al.*, 2004). Furthermore, it has been described that VEGFC and VEGFD can also bind VEGFR-2 (Joukov *et al.*, 1996; McColl *et al.*, 2007; Das and Skobe, 2008; Duong *et al.*, 2012). In addition, VEGFR-3 has also been detected in the angiogenic blood endothelium of different tumours (Valtola *et al.*, 1999; Witmer *et al.*, 2001).

Also FGF-2, PDGF, angiopoietins (Ang-1 and Ang-2), hepatocyte growth factor (HGF) and insulin like growth factor (IGF-1 and IGF-2) have been reported to induce lymphangiogenesis (Cao, 2005; Van der Auwera *et al.*, 2006; Cao, 2008; Da *et al.*, 2008; Das and Skobe, 2008; Stacker and Achen, 2008; Ran *et al.*, 2010; Duong *et al.*, 2012). Nevertheless, it has been reported that lymphangiogenesis can occur without induction of angiogenesis (Cao, 2005). As with angiogenesis, the lymphangiogenic vessels are disorganized without a hierarchical vascular pattern and are not homogenously distributed within the tumour. A large number of the vessels is functionally abnormal (Isaka *et al.*, 2004; Cao, 2008; Li *et al.*, 2012).

To date, very little is known on lymphangiogenesis in canine tumours. Recent research identified VEGFC, VEGFD and VEGF-3 in CMTs (Qiu *et al.*, 2008; Clemente *et al.*, 2013).

1.3.3. *Vascular metastasis*

Metastasis remains the cause of death of more than 90% of cancer related deaths from solid tumours in human medicine (Gupta and Massague, 2006). Almost half of the patients with malignant CMTs die or are euthanized within 1 year of surgery because of tumour recurrence or metastasis (Misdorp and Hart, 1979; Graham and Myers, 1999). Lymphatic metastasis is considered as the main route for canine and human breast carcinomas, although haematogenous metastasis can also occur (Figure 1-6) (Perez Alenza *et al.*, 1997; Pantel and Woelfle, 2004; Sleeckx *et al.*, 2011; Rasotto *et al.*, 2012; Santos *et al.*, 2013). The lymphatic vessels drain interstitial fluid, containing tumour cells, away from the tumour (Jain, 1989; Thiele and Sleeman, 2006; Rovenska and Rovensky, 2011). Tumour lymphangiogenesis might enhance metastasis by increasing the number of lymphatic vessels, which augments the contact surface area between the invading cancer cells and the lymphatic endothelium (Karpanen *et al.*, 2001; Skobe *et al.*, 2001b; Saharinen *et al.*, 2004). In addition, lymphatic vessels may increase in diameter, which can encourage the spread of invaded tumour cells (Achen and Stacker, 2008). It should be emphasized, that for lymphatic metastasis the newly formed lymphatic vessels are essential, but

not the sole element in a complex sequence of events leading to metastasis. The newly formed lymph node metastases can in turn shed tumour cells into the efferent lymphatics, through which the tumour cells can be transported via the lymphatic system to the blood circulation and spread to different tissues. Nevertheless, it is possible that distant metastasis can be found without detectable lymph node metastasis (Sleeman, 2000; He *et al.*, 2002; Taubert *et al.*, 2004; Sleeman *et al.*, 2009).

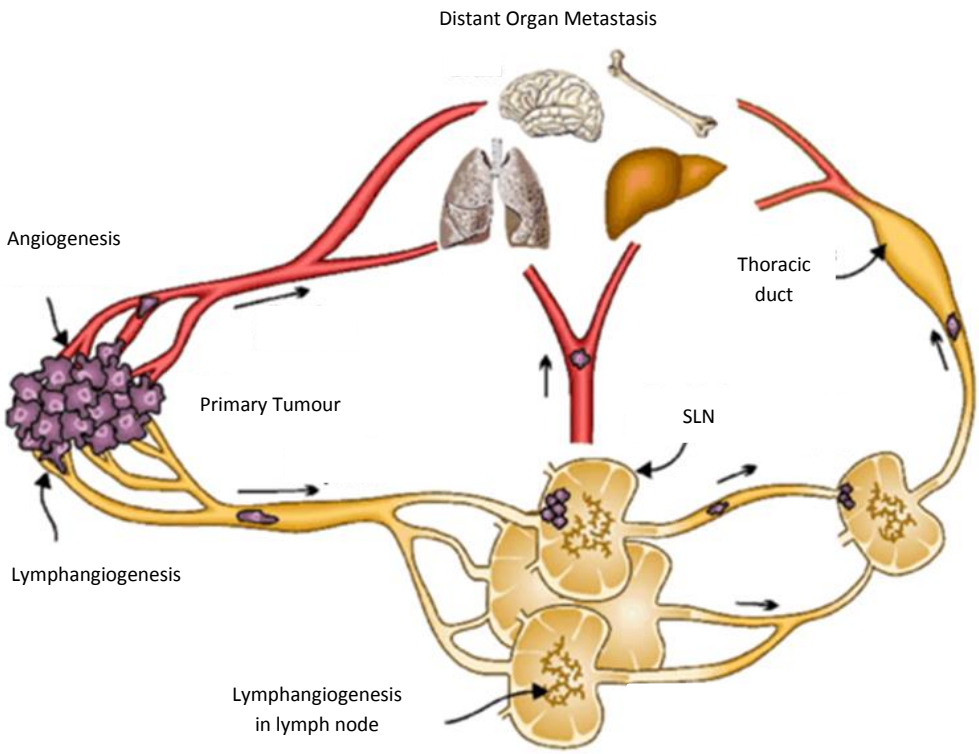


Figure 1-6 *Schematic representation of potential routes of metastasis via the lymphatic vasculature (yellow), blood vessels (red) and lymph nodes. SLN = sentinel lymph node (Adapted from Achen and Stacker, 2008)*

Initially, it was suggested that only the pre-existing lymphatics surrounding the tumour margin were involved in metastasis and that intratumoural lymphatics were absent (Carmeliet and Jain, 2000; Vleugel *et al.*, 2004; Agarwal *et al.*, 2005). Evidence

was based on the failure of identifying intratumoural lymphatics by injecting tracers into lymphatics or by labelling the lymphatics with immunohistochemical markers such as lyve-1. The heterogenic distribution of the lymphatic vessels, collapse of the intratumoural lymphatics due to the increased pressure and mechanical stress generated by the proliferating tumour cells together with the inability of certain immunohistochemical staining methods, can explain this failure to show intratumoural lymphatics in some tumours (Padera *et al.*, 2002; Van der Auwera *et al.*, 2005). However, different more recent studies in human breast cancer did show the induction of new lymphatic vessels in and around the tumour and acknowledge the prognostic value of peritumoural and intratumoural lymphatic vessels for the patient's survival and metastatic behaviour of the tumour (Cao, 2005; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009; Mohammed *et al.*, 2009; Ran *et al.*, 2010; Kandemir *et al.*, 2012). Consequently, dissemination of tumour cells from the primary tumour to the lymphatic system occurs by both peritumoural and intratumoural lymphatics (Skobe *et al.*, 2001b; Beasley *et al.*, 2002; Stacker *et al.*, 2002; Achen *et al.*, 2005).

The basis of the current knowledge of the metastatic process is still the 'Seed and Soil' hypothesis, proposed by Paget (Paget, 1889). He suggested that tumour cells ('seed') selectively colonized the 'soil' of distant organs with an environment favourable for survival and proliferation. Many years later, it has been shown that this 'soil', the pre-metastatic niche, undergoes a series of changes initiated by different growth factors that are secreted by primary tumour cells and associated stromal and inflammatory cells to prime it for the arrival of tumour cells (Skobe *et al.*, 2001a; Kaplan *et al.*, 2005; Psaila *et al.*, 2006; Psaila and Lyden, 2009). Genetic background and activation of specific chemokines/cytokines and proteases may direct metastases to a specific organ. For example breast cancer cells expressing the chemokine receptor CXCR4 home to organs with high levels of its ligand SDF-1 (stromal derived factor, CXCL12), which can be found in bone marrow, lung and liver. In addition, specialised breast carcinoma associated fibroblasts can attract CXCR4+ tumour cells via expression of CXCL12 and also stimulate tumour angiogenesis

(Kaplan *et al.*, 2005; Psaila *et al.*, 2006; Joyce *et al*., 2009). Different studies demonstrated the induction of angiogenesis and lymphangiogenesis in these pre-metastatic sites and their involvement in the metastatic spread of tumour cells, as they make the pre-metastatic niche more favourable for incoming tumour cells and facilitate further metastatic spread to distant organs (Guidi *et al.*, 2000; Qian *et al.*, 2006; Hirakawa *et al.*, 2007; Achen and Stacker, 2008; Cueni and Detmar, 2008; Hirakawa, 2009). A significant correlation between sentinel lymph node lymphangiogenesis and distant lymph node metastasis was found in human breast cancer patients (Van den Eynden *et al.*, 2007b).

1.3.4. Blood and lymphatic endothelial cell markers

One of the most important methodical problems for the study of lymphatic vessels is the differentiation with blood vessels, as both vessel types consist of endothelial cells with similar characteristics (Van den Eynden *et al.*, 2006). The distinction between blood and lymphatic vessels demands highly specific markers (Baluk and McDonald, 2008). The most commonly used IHC blood vessel markers are CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1), CD34 (hematopoietic progenitor cell antigen 34), and the von Willebrand factor (vWf, factor VIII). **CD31** is a transmembrane glycoprotein adhesion molecule expressed by platelets, megakaryocytes and endothelial cells. It stains small and larger blood vessels as well as immature vessels, yet it can also label fibroblasts and some inflammatory cells e.g. plasma cells (Parums *et al.*, 1990; Uzzan *et al.*, 2004; Pusztaszeri *et al.*, 2006; Ordonez, 2012). **vWf** is a glycoprotein that mediates platelet adhesion and binds and stabilises factor VIII. It is stored in endothelial cell Weibel-palade bodies, and mainly stains mature vessels (Uzzan *et al.*, 2004; Pusztaszeri *et al.*, 2006). **CD34** is a transmembrane glycoprotein expressed by endothelial cells and hematopoietic stem cells and may function in part as a cell trafficking molecule for leukocytes and hematopoietic stem cells. It has the same characteristics as CD31 but appears to give a more intense staining (Uzzan *et al.*, 2004; Pusztaszeri *et al.*, 2006; Nielsen and McNagny, 2009). These blood vessel IHC markers have successfully been applied on

human (Hansen *et al.*, 1998; Guidi *et al.*, 2000; Uzzan *et al.*, 2004; Van der Auwera *et al.*, 2005) and canine tissues (Graham and Myers, 1999; Restucci *et al.*, 2000; Maiolino *et al.*, 2001; Restucci *et al.*, 2003; Martano *et al.*, 2004; Luong *et al.*, 2006; Tonar *et al.*, 2008; Wolfesberger *et al.*, 2008; Lavalle *et al.*, 2009; Clemente *et al.*, 2010a; Im *et al.*, 2011; Cuitino *et al.*, 2012; Wolfesberger *et al.*, 2012; Yhee *et al.*, 2012). All three markers can cross-react at a varying degree with lymphatic vessels, making the combination with a lymphatic marker indispensable (Uzzan *et al.*, 2004; Ordonez, 2012).

Several markers for lymphatic vessels in human medicine have been studied during the last decades. VEGFR-3, lyve-1, prox-1 and podoplanin are currently used as markers for lymphatic vessels (Ordonez, 2012). **VEGFR-3** is a tyrosine kinase that is predominantly expressed in lymphatic endothelial cells in adult tissue and was used in earlier lymphatic vessel studies. It can also be found in fenestrated capillaries of several organs and in tumour associated angiogenic blood vessels, which makes this marker not reliable for discriminating between blood and lymphatic vessel endothelial cells. In addition, controversy exist on the expression of VEGFR-3 on tumour cells (Reis-Filho and Schmitt, 2003; Al-Rawi *et al.*, 2005a; Van der Auwera *et al.*, 2006; Sarli *et al.*, 2007; Baluk and McDonald, 2008; Petrova *et al.*, 2008; Sleeman *et al.*, 2009; Holopainen *et al.*, 2011). Another marker is lymphatic vessel endothelial hyaluronan receptor I (**lyve-1**), which is an integral membrane glycoprotein that functions as a receptor for hyaluronan, a ubiquitous extracellular glycosaminoglycan involved in cell migration and differentiation. However, lyve-1 is also expressed in the discontinuous endothelia of liver and spleen sinusoids, pulmonary capillaries and in macrophages. Furthermore, it has been observed that the expression of lyve-1 can be down-regulated in response to inflammation in some tissues, is less strongly expressed on collecting lymphatics than on initial lymphatics and can be absent in some tumour associated lymphatics (Banerji *et al.*, 1999; Mouta Carreira *et al.*, 2001; Reis-Filho and Schmitt, 2003; Van der Auwera *et al.*, 2004; Van der Auwera *et al.*, 2006; Sleeman *et al.*, 2009). The homeodomain protein prospero-related homeobox I **(prox-1**), which is a nuclear transcription factor, is required for the regulation of

lymphatic vascular development from early stages of embryonic veins. Prox-1 expression persists in adult lymphatic endothelium and can also be found in other cell types than lymphatic endothelial cells: non-endothelial cells in the lens, heart, liver, pancreas and nervous system. In contrast to the other lymphatic markers, prox-1 immunoreactivity is nuclear (Wigle and Oliver, 1999; Stacker *et al.*, 2002; Wilting *et al.*, 2002; Reis-Filho and Schmitt, 2003; Van der Auwera *et al.*, 2006; Ordonez, 2012). **Podoplanin**, a surface glycoprotein that is expressed on lymphatic endothelial cells may play a role in cell adhesion. It can also be found on osteoblastic cells, lung alveolar type I cells, kidney podocytes, stromal reticular cells and follicular dendritic cells. It may also be expressed on tumour cells and tumour associated fibroblasts (Reis-Filho and Schmitt, 2003; Schacht *et al.*, 2005; Van der Auwera *et al.*, 2006; Baluk and McDonald, 2008; Kawase *et al.*, 2008). Like lyve-1, podoplanin seems to be less expressed in collecting lymphatic vessels (Van der Auwera *et al.*, 2006; Sleeman *et al.*, 2009). The most recently described monoclonal antibody **D2-40** recognises human podoplanin. This antibody has been shown to be a highly selective marker of lymphatic endothelium in normal and neoplastic tissues (Kahn and Marks, 2002).

D2-40 antibody can also react with the basal epithelial cell layers of the epidermis and of human breast and prostate gland (Kahn *et al.*, 2002; Van der Auwera *et al.*, 2006; Marinho *et al.*, 2008; Britto *et al.*, 2009). A comparative study of antibodies directed at lyve-1, prox-1, podoplanin and D2-40 on human breast carcinomas identified D2-40 as the best marker of lymph vessels in breast cancer (Van der Auwera *et al.*, 2005).

All lymphatic vessel markers have been applied successfully to human tissues (Hansen *et al.*, 1998; Guidi *et al.*, 2000; Reis-Filho and Schmitt, 2003; Duff *et al.*, 2007; Baluk and McDonald, 2008). In contrast, only a few veterinary studies have applied lymphatic markers. Lyve-1 and prox-1 were used in an investigation of feline lymphangiosarcoma (Galeotti *et al.*, 2004; Sugiyama *et al.*, 2007) and prox-1 has been used in a study of equine normal tissue and lymphangioma (Staszyk *et al.*, 2005;

Junginger *et al.*, 2010) and to investigate normal canine tissue (Saito *et al.*, 2006; Martin *et al.*, 2010).

1.4. Fibrotic Foci

First proposed by Hasebe in 1996 as an indicator of tumour aggressiveness, but already described more than a decade earlier (Fisher *et al.*, 1983), a fibrotic focus (FF) is a scar-like area in the centre of a carcinoma and can be regarded as a focus of exaggerated reactive stroma formation (Hasebe *et al.*, 1996). The radially expanding fibrosclerotic appearance of a FF and its composition strongly resemble that of a wound-healing process. The main components of a FF are fibroblasts and collagen fibre in variable amounts, which are arranged differently in a FF compared to normal breast cancer stroma or the surrounding non-FF tumour stroma. These fibroblasts are highly proliferative and produce extra cellular matrix (ECM). As a consequence, a FF contains variable amounts of collagen and elastic fibres (Hasebe *et al.*, 2001; Van den Eynden *et al.*, 2007a). The degree of fibrosis is classified into three grades according to the following criteria; grade 1: a large number of fibroblasts with a small amount of collagen fibres, grade 3: mainly composed of collagen fibres, mostly hyalinised and grade 2: intermediate between 1 and 3, with fibroblasts and collagen fibres intermingled in varying ratios. To what extent ECM in a FF differs from the ECM in normal breast stroma or surrounding tumour stroma remains unknown (Hasebe *et al.*, 1996). A FF is located within the tumour, surrounded by a more cellular zone of invading tumour cells and it occupies various percentages of the tumour area. However, when the FF is 3 mm or smaller, tumour cells are infrequently seen within it (Van den Eynden *et al.*, 2007a).

Hypoxia, which occurs when a tumour outgrows its blood supply, is the driving force for the formation of a FF. The presence of a FF was correlated with the increased expression of hypoxia markers HIF1-α and CA9, both in carcinoma cells and intratumoural fibroblasts. Hypoxia leads to the proliferation and activation of stromal fibroblasts with abundant and disorganized deposition of EC matrix proteins and to the induction of angiogenesis. These stromal changes are involved in tumour

progression, increased hypoxia and metastasis (Colpaert *et al.*, 2001b; Van den Eynden *et al.*, 2007a; Van den Eynden *et al.*, 2008). A significant correlation between high angiogenesis and the presence of a FF was found (Hasebe *et al.*, 1998; Jitsuiki *et al.*, 1999; Hasebe *et al.*, 2000; Colpaert *et al.*, 2001a; Colpaert *et al.*, 2003). However, next to blood vessels, a FF also contains lymphatic vessels and in tumours with a FF, tortuous newly formed lymphatic vessels are often found in vascular hotspots at the border of the FF together with newly formed blood vessels. Although VEGFC and VEGFD are thought to be the most important lymphangiogenic factors, no difference in expression of VEGFC in tumours with a (large) FF was observed and VEGFD was even decreased. However, as described before, VEGFA is not only an angiogenic, but also a lymphangiogenic factor and VEGFR2 is also expressed on lymphatic endothelial cells (Colpaert *et al.*, 2001b; Colpaert *et al.*, 2003; Van den Eynden *et al.*, 2007a).

The importance of a FF in invasive ductal carcinoma has been analysed extensively during the last two decades. Most studies found that the presence of a FF was associated with higher histological grade, higher frequency of lymph node metastases, more distant metastases and higher proliferative activity. Thus, it is an important factor for accurately predicting the outcome of patients with invasive ductal breast carcinoma, both for tumour recurrence and patient's death. In addition, the presence of a FF makes it possible to identify cancers which may behave aggressively in otherwise low risk groups (Hasebe *et al.*, 1996; Hasebe *et al.*, 1997; Hasebe *et al.*, 1998; Koyama *et al.*, 1999; Hasebe *et al.*, 2000; Colpaert *et al.*, 2001a; Colpaert *et al.*, 2001b; Hasebe *et al.*, 2002; Baak *et al.*, 2005; Hasebe *et al.*, 2008; Tamura *et al.*, 2009; Hasebe *et al.*, 2011a). In addition, the presence of atypical stromal fibroblast especially in a fibrotic focus are associated with tumour recurrence and tumour-related death in patients with IDCs (Hasebe *et al.*, 2011b; Hasebe *et al.*, 2011c). These findings were not confirmed in a recent study, where a FF showed an inverse relationship with for example tumour cell proliferation. In this study where FF size was not correlated to any histologic parameter or biomarker expression, the mean FF size was smaller and the mean tumour size was bigger than those in

previously reported studies (Mujtaba *et al.*, 2013). Further investigation to confirm these results needs to be performed.

Fibrotic foci can be easily recognised on standard HE stains, which makes them more practical for routine use than immunohistochemistry, DNA analysis or molecular genetics (Van den Eynden *et al.*, 2007a). However, pre-operative detection of a FF could be important in planning the type of therapy as patients with tumours that contain a FF, will benefit from a more aggressive approach. Breast sonograms showed a focal increased echogenicity in 75% of the patients with a FF in IDCs that directly correlated with the FF seen histologically. Possible explanations for the lack of visualisation in the remaining 25% are: oedema within the FF, presence of necrosis within FF, evolving or small FF with residual tumour cells present, relative size FF (Oken *et al.*, 2005). Furthermore, it was recently reported that MR mammography can recognise carcinomas with a FF as well as a good estimate of the diameter of the FF (Van Goethem *et al.*, 2012).

Fibrotic foci have also been described in other tumours e.g. male breast cancer, colorectal carcinoma, pancreatic ductal adenocarcinoma, gastric cancer and an association with tumour aggressiveness was described (Nishimura *et al.*, 1998; Couvelard *et al.*, 2005; Mitsunaga *et al.*, 2005; Okamoto *et al.*, 2008; Kornegoor *et al.*, 2012).

A recent study showed the presence of hypoxia in CMTs (Mees *et al.*, 2011). To the best of our knowledge, no data on the presence of FF in CMTs is present in veterinary literature.

1.5. Comparative Oncology

The value of comparative oncology, which is the study of spontaneously occurring tumours in domestic animals as models for human disease, has already been recognised during the last century and has gained increasing interest during the last decade (Cotchin, 1962; Weijer *et al.*, 1972; Strandberg and Goodman, 1974; Cotchin, 1976; Withrow *et al.*, 1991; Knapp and Waters, 1997). This approach can make important contributions to the understanding and evolution of human oncology in different fields such as basic tumour biology, immunology, therapeutic response, etc. for a variety of cancers, as naturally occurring tumours in pet dogs have clinical and biological similarities to human cancers that are difficult to replicate in other model systems (Gordon *et al.*, 2009). For example, many anti-cancer agents fail in human cancer patients because of unacceptable toxicity or poor efficacy, even after absence of toxicity and evidence of drug efficacy in mouse and healthy dog preclinical models (Gordon *et al.*, 2009).

A historical limitation to the widespread use and integration of the comparative approach has been a lack of infrastructure to coordinate animal health professionals with the human oncology community, drug developers and basic scientists (Gordon *et al.*, 2009). The founding of the Comparative Oncology Program (COP) in the USA, the Centre for Clinical and Comparative Oncology C3O in Sweden and the LUPA-project in Europe among others, created opportunities to the widespread use of animals with spontaneously developing cancers as models for human disease (Paoloni and Khanna, 2008; Lequarre *et al.*, 2011).

1.5.1. Murine animal model

Murine studies are extremely useful for analysing the biology of pathways involved in cancer initiation, promotion and progression. The short duration, reduced follow-up time, low costs and a high repeatability make them ideal animal models for preclinical studies (Porrello *et al.*, 2004; Gordon *et al.*, 2009). However, the translation of these results to human clinical trials is complicated by the differences between the murine cancer models and cancer seen in human patients including

growth over long periods of time, genomic instability, and the heterogeneity of tumour cells and their surrounding microenvironment. In addition, the tumour host relationship is biased by the partially or completely compromised immune system in nude or scid mice respectively (Porrello *et al.*, 2004; Rusk *et al.*, 2006). The complex biology of tumour development, cancer recurrence and metastasis, and responses to therapy are not adequately reproduced in the conventional high inbred murine models, due to the lack of intratumoural and inter-individual heterogeneity (Strandberg and Goodman, 1974; Rusk *et al.*, 2006; Gordon *et al.*, 2009; Pinho *et al.*, 2012). As a result, many novel anticancer agents fail in human cancer patients because of unacceptable toxicity or poor efficacy despite evidence of drug efficacy in mouse models of cancer (Khanna and Vail, 2003; Gordon *et al.*, 2009). For example, tumour angiogenesis, which is an important factor in many human tumours, can be completely reversed in mice, leading to tumour regression or dormancy. However, some studies in humans failed to confirm similar results (Porrello *et al.*, 2004). In addition, the non-cytotoxic mechanism of action of some anti-angiogenic therapies is slower and therefore difficult to assess in the more rapidly progressive mouse models of cancer (Rusk *et al.*, 2006).

1.5.2. Canine animal model: purpose-bred Beagles

Typically, toxicity evaluation is based on preclinical studies in a rodent and a non-rodent species (Dixit and Boelsterli, 2007). Purpose bred dogs, Beagles in particular, are largely used in pharmacokinetic and toxicological studies (Porrello *et al.*, 2004; Gordon and Khanna, 2010). These healthy dogs receive an investigational agent in a controlled setting for assessment of pharmacokinetic and pharmacodynamic parameters and toxicity (Gordon and Khanna, 2010). However, this toxicity testing in healthy animals may have a poor predictive value for some agents and diseases (Dixit and Boelsterli, 2007). Dogs with naturally occurring tumours can have systemic diseases and consequences of cancer and can suffer from age, other diseases and the effects of concomitant medications, which is very difficult to replicate (Khanna and Vail, 2003; Gordon *et al.*, 2009). This complex situation may result in differences in toxicities and pharmacokinetics from that seen in healthy young colony bred

research dogs and may serve as an ideal model for the anticancer activity of novel agents (Gordon and Khanna, 2010; Khanna, 2011).

1.5.3. Canine animal model: companion dogs with spontaneously occurring cancer

Studies in pet dogs with naturally occurring tumours show many advantages. Cancer is the number one cause of death in dogs (Khanna and Hunter, 2005). In an autopsy series of 2000 dogs, 23% of all dogs regardless of age, and 45% of dogs of more than 10 years of age, died of cancer (Bronson, 1982). This large canine cancer patient population worldwide is an important underused study population as many of these companion animal owners frequently seek out specialized care and treatment, motivated by the hope of prolonged quality of their animals' life (Rowell *et al.*, 2011). Enrolled in a comparative oncology study they can benefit from the latest developed therapies, often with considerable funding (Porrello *et al.*, 2004). Moreover, dogs show resemblance with humans concerning anatomy and physiology, particularly with cardiovascular, urogenital, nervous and musculoskeletal systems. In addition, the hepatic enzyme homology of dogs is much more similar to humans than to rodents, which can be of high importance in drug research (Pinho *et al.*, 2012). Furthermore, dogs have a large breed diversity and show strong genetic similarities to humans. For many gene families, most notably those associated with cancer, the similarities are significantly higher between dog and man than between mouse and man (De Vico *et al.*, 2005; Paoloni and Khanna, 2007). Most cancer associated genetic alterations that have been identified in human cancers, have been shown to play a role in canine cancers (Paoloni and Khanna, 2007; Paoloni and Khanna, 2008).

Many tumours including human and canine osteosarcoma, prostate tumour, melanoma, non-Hodgkin lymphoma, head and neck carcinoma, soft tissue sarcoma, as well as mammary tumours, show strong similarities for morphological, biological and molecular characteristics (Strandberg and Goodman, 1974; Cotchin, 1976; De Vico *et al.*, 2005; Khanna and Hunter, 2005; Khanna *et al.*, 2006). Also, recurrent and metastatic disease, response and/or resistance to different therapies and neo-angiogenesis patterns, were seen in canine tumours (Khanna *et al.*, 2006; Paoloni and

Khanna, 2007; De Vico, 2008; Paoloni and Khanna, 2008; Gordon *et al.*, 2009; Pang and Argyle, 2009). The tumour growth in both humans and companion animals occurs in an intact immune system with a syngeneic host and tumour microenvironment and initiation and progression are influenced by similar factors including age, nutrition, sex and reproductive status (Hansen and Khanna, 2004; De Vico *et al.*, 2005; Khanna, 2008; Paoloni and Khanna, 2008; Gordon *et al.*, 2009). Furthermore, interindiviual and intratumoural heterogeneity are present (Khanna *et al.*, 2006; Khanna, 2008). Dogs can also serve as sentinels for environmental cause of disease as they develop tumours spontaneously after years of exposure to the same environmental oncogenic factors of the owners (De Vico *et al.*, 2005; Gordon *et al.*, 2009; Marconato *et al.*, 2009a; Andrade *et al.*, 2010). In particular for mammary tumours many similarities are present. Dogs develop mammary cancer spontaneously with comparable morphology, epidemiology, genetics, and clinical features as human breast cancer (Lindblad-Toh *et al.*, 2005; Uva *et al.*, 2009; Mohammed *et al.*, 2011; Queiroga *et al.*, 2011). Both show spontaneous development, hormonal dependence, metastatic behaviour towards regional lymph nodes and lungs. Moreover, dogs are the only documented species besides humans that spontaneously develop inflammatory mammary carcinoma (Porrello *et al.*, 2006). Also on molecular level, canine mammary tumours seem to mimic human breast cancer as similarities are found concerning the overexpression of steroid receptors, proliferation markers, epidermal growth factor, p53 suppressor gene mutations, metalloproteinases, cyclooxygenases, BRCA and ERbB among many others (Queiroga *et al.*, 2011; Rivera and von Euler, 2011; Pinho *et al.*, 2012). However, comparative studies on angiogenesis are scarce or even absent for lymphangiogenesis.

The larger body size of canines, compared to rodents, facilitates the serial collection of blood, urine, bone marrow and, very importantly, of tumoural and normal tissue biopsies from the same patient before, during and after exposure to an investigational agent (Hansen and Khanna, 2004; Gordon *et al.*, 2009; Pinho *et al.*, 2012) This sequential sampling allows to study tumoural and pharmacodynamic

endpoints or biomarkers that can be uniquely correlated to drug exposure, and therapeutic response in ways that are often difficult or unacceptable in human trials (Gordon *et al.*, 2009).

Comparative oncology studies can provide information on toxicity, response, pharmacokinetics, pharmacodynamics, dose, regimen as well as new insights in cancer associated genes, tumour molecular biology and evaluation and development of novel diagnostic, prognostic and therapeutic applications that will benefit both dogs and human cancer patients (De Vico *et al.*, 2005; Porrello *et al.*, 2006; Pinho *et al.*, 2012). However, comparative oncology studies will have different endpoints depending on the phase in the drug development pathway. For agents that are still in preclinical stage, companion dog studies may provide efficacy, dose and toxicity data to support initiation of human phase I studies. For agents that are in a later stage, clinical anti-tumour activity (decrease in size or time to recurrence) will become more important (Hansen and Khanna, 2004; Khanna and Gordon, 2009). As a result the approval of new drugs can be accelerated and costs and risks can be decreased by reducing the number of drugs entering each phase of drug development and increasing the success rate in phase II trials as an integrated approach (De Vico *et al.*, 2005; Khanna and Hunter, 2005; De Vico, 2008).

It is important to realize that enrolled diseased dogs and cats should be considered primarily as animal patients, a position rather different from that of mere being considered an animal model (De Vico, 2008). The pet dog animal model can be integrated as an extra preclinical study to provide additional information on the efficacy and toxicity before moving on to a phase I human clinical study, but can also be useful in between and during the different phases of human clinical trials (Figure 1-7) (Gordon and Khanna, 2010). In contrast to humans, gold standard treatment often lacks for many canine tumours. This means that novel agents can be offered to pet dogs as single agents before any conventional treatment has been provided or that they can be added to treatment regimens such as chemotherapy and radiation therapy to determine optimal therapeutic combination. This permits evaluation of

new drugs early in the development life of the drug in different settings. These studies are often hindered in humans by the fact that the combination therapy of interest may be contrary to accepted treatment standards. In addition, the canine cancer patients are generally less heavily pre-treated compared to most human cancer patients participating in human clinical trials (Paoloni and Khanna, 2008; Sahora *et al.*, 2012).

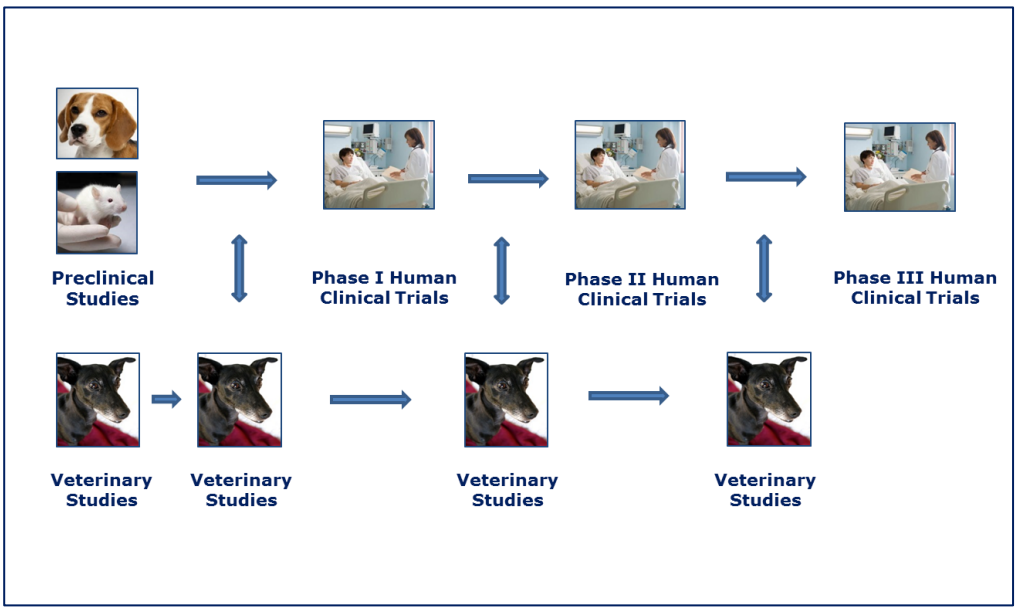


Figure 1-7 *The integration of pet dogs in the drug development pathway (Adapted from https://ccrod.cancer.gov/confluence/display/CCRCOPWeb/Development+Path)*

An additional argument for integrating the comparative approach is the improvement of the drug development pathway of new anti-cancer agents, e.g. molecular targeted agents (Gordon *et al.*, 2009; Gordon and Khanna, 2010). The attrition rate, even in late phase studies, for molecular targeted agents that enter clinical trials is high. This suggests that preclinical development may not always be successful in predicting activity and safety of these targeted agents that can modify the outcome of human cancer. Both in vitro and in vivo preclinical models, such as cell lines and rodents, have their limitations due to their dissimilarities to

spontaneous human cancer (Gordon *et al.*, 2009; Ocana *et al.*, 2011). In addition, one of the aims of phase I studies, the identification of a maximum tolerated dose for phase II studies, is based on the assumption that therapeutic and toxic effects are related to each other and are caused by the same mechanism of action. This may not be the case for molecular targeted agents, which may have different mechanisms of the therapeutic and toxic effects and might be more effective at a lower dose for a longer period of time. Phase II studies normally have response rate of the tumour as primary endpoint. Some tumours may not respond appropriately to the cytostatic effect of some newly targeted agents within a certain exposure period. Studies that are longer in time and that use time to progression or progression-free survival may be required to adequately evaluate these agents (Gordon and Khanna, 2010). Furthermore, the integration of comparative oncology studies to assess the efficacy in the adjuvant or minimal residual disease setting may prioritize those agents most likely to be effective as Phase III human cancer agents (Gordon *et al.*, 2009).

Obviously, there are some limitations to the comparative animal model. Studies take longer and have a higher cost compared to mouse models. Depending on the type of agent and study questions asked, study entry requirements may be limited to pet dogs of specific age, sex, size and/or medical conditions. In some cases, use of the canine cancer patients is not possible for some molecular targeted agents when the interaction with the agent and the targeted molecules differs between man and dog (Hansen and Khanna, 2004; Paoloni and Khanna, 2008; Gordon and Khanna, 2010).

It is important to realise that non-human clinical studies that include pet dogs with cancer cannot replace the conventional and necessary studies with inbred purpose-bred research mice and Beagles, but they can be an intermediary between conventional preclinical models and the human clinical trial and between the different clinical trial phases. Data gathered in the conventional studies may even be used in the design of studies with dogs with spontaneously occurring tumours (Hansen and Khanna, 2004; Khanna and Gordon, 2009). Not all agents can or should be evaluated in tumour bearing dogs and not all questions can be answered using

this approach. Strategic inclusion of pet dog studies within a preclinical and clinical development path can prevent any delays in the conduct or completion of human clinical trials.

In summary, the biological complexity of naturally occurring cancers in pet dogs, their size and strong similarities to human cancers, together with a strong interest of pet dog owners for cancer treatment, provides an opportunity to develop a comparative and integrated approach to cancer drug development. This approach can benefit both man and dog, as the approval of effective new cancer drugs will accelerate and as dogs can benefit from state-of-the art treatment protocols that otherwise are not available in veterinary medicine (Paoloni *et al.*, 2009; Gordon and Khanna, 2010). However, in the cancer research community, the awareness of naturally occurring cancer models is relatively limited. Broader awareness of these naturally occurring cancer models is needed, which can create opportunities for collaboration and interaction between comparative veterinary oncologists and the cancer research community.

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CHAPTER 2: *Aims*

The goal of this study is to gain information on the presence and characteristics of blood vessels, lymphatic vessels and fibrotic foci in canine mammary tumours as prognostic markers for the canine patient. These factors can be evaluated on HE and immunohistochemical stained tumour samples, which makes them reasonably cheap and practical histological parameters. Moreover, dogs with canine mammary tumours were assessed in view of being a possible comparative oncology model for anti-angiogenic and anti-lymphangiogenic therapy. The scientific aims of this thesis therefore are:

1 develop immunohistochemical protocols to identify and to differentiate blood and lymphatic vessels in tumoural and normal canine mammary gland tissue (***Chapter 3***).

2 define ***a)*** the morphological characteristics of **lymphatic vessels** in benign and malignant canine mammary tumours and in normal canine mammary gland tissue and ***b)*** the association of these characteristics with the clinical features of the patients (***Chapter 4***).

3 study ***a)*** the morphological characteristics of **blood vessels** in benign and malignant canine mammary tumours and in normal canine mammary gland tissue and ***b)*** the association of these characteristics with the clinical features of the patients (***Chapter 5***).

4 examine **proliferating** lymphatic endothelial cells and blood endothelial cells (***Chapter 4 and 5 respectively***).

5 investigate ***a)*** the morphological characteristics of **fibrotic foci** in malignant canine mammary tumours and ***b)*** the association of these characteristics with the clinical features of the patients (***Chapter 6)***.

CHAPTER 3: Evaluation of immunohistochemical markers of lymphatic and blood vessels in canine mammary tumours

Based on:

Sleeckx N., Van Brantegem L., Fransen E., Van den Eynden G., Casteleyn C., Veldhuis Kroeze E., Van Ginneken C. (2013) Evaluation of Immunohistochemical Markers of Lymphatic and Blood Vessels in Canine Mammary Tumours. *Journal of Comparative Pathology*, **148,** 307-17.

3.1. Abstract

Canine mammary tumours (CMTs) are the most common neoplasms in intact female dogs. Bitches with spontaneously arising CMTs represent a promising animal model for human breast cancer research. The aim of the present study was to develop an immunohistochemical protocol for the identification of blood and lymphatic vessels in CMTs. Antibodies specific for human lymphatic vessels (prox-1, lyve-1, podoplanin and D2-40) and blood vessels (von Willebrand factor [vWf], CD31 and CD34) were utilised. Serial sections of 18 samples (eight samples of normal canine mammary tissue, five benign and five malignant CMTs) were examined. Antibodies specific for podoplanin, D2-40 and CD34 showed no immunoreactivity with canine tissue. Prox-1 and CD31 were determined to be the most suitable markers for lymphatic and blood vessels, respectively.

3.2. Introduction

Mammary tumours are the most common neoplasms of women and bitches (MacEwen and Withrow, 1996; Sleeckx *et al.*, 2011). Canine mammary tumours (CMTs) are solid tumours that consist of tumour cells and tumour-associated stroma. The latter contains blood and lymphatic vessels (Folkman, 1986; Van der Auwera *et al.*, 2005). During tumour growth both angiogenesis and lymphangiogenesis are induced. The formation of these new blood and lymphatic vessels, originating from the pre-existing vascular network, is essential for tumour growth and metastasis (Folkman, 1986; Fox *et al.*, 1996; Saaristo *et al.*, 2000; Al-Rawi *et al.*, 2005; Sleeman *et al.*, 2009; Holopainen *et al.*, 2011). As occurs in human medicine (Weidner *et al.*, 1991; Uzzan *et al.*, 2004; Van der Auwera *et al.*, 2006), a relationship between angiogenesis, malignancy and prognosis has recently been shown in veterinary medicine for different tumour types including CMTs (Griffey *et al.*, 1998; Graham and Myers, 1999; Restucci *et al.*, 2000; Maiolino *et al.*, 2001; Restucci *et al.*, 2003; Martano *et al.*, 2004; Preziosi *et al.*, 2004; Luong *et al.*, 2006; Lavalle *et al.*, 2009). However, to our knowledge,

characterization of lymphangiogenesis and its correlation with clinical features is still lacking for CMTs.

One of the most important methodical problems for the histopathological study of blood and lymphatic vessels is the differentiation between the two vessel types, which both consist of endothelial cells with similar characteristics (Van den Eynden *et al.*, 2006). The distinction between blood and lymphatic vessels demands highly specific immunohistochemical markers (Baluk and McDonald, 2008). The most commonly used blood vessel markers are CD31, CD34 and the factor VIII-related antigen or von Willebrand factor (vWf). Markers for lymphatic vessels include the lymphatic vessel endothelial hyaluronan receptor (lyve-1), the homeodomain protein prospero-related homeobox I (prox-1), which is a transcription factor, the glomerular podocyte membrane protein podoplanin, and D2-40, which recognises podoplanin (Schacht *et al.*, 2005). Both blood and lymphatic vessel markers have been applied successfully to human tissues (Hansen *et al.*, 1998; Guidi *et al.*, 2000; Reis-Filho and Schmitt, 2003; Duff *et al.*, 2007; Baluk and McDonald, 2008).

Most studies of canine blood vessels have used vWf (Graham and Myers, 1999; Restucci *et al.*, 2003; Martano *et al.*, 2004; Luong *et al.*, 2006; Tonar *et al.*, 2008; Wolfesberger *et al.*, 2008), CD31 (Restucci *et al.*, 2000; Maiolino *et al.*, 2001; Luong *et al.*, 2006; Lavalle *et al.*, 2009; Clemente *et al.*, 2010) and CD34 (Clemente *et al.*, 2010; Gillespie *et al.*, 2011) as markers of endothelium. In contrast, only a few studies have applied lymphatic markers. Lyve-1 and prox-1 were used in an investigation of feline lymphangiosarcoma (Galeotti *et al.*, 2004; Sugiyama *et al.*, 2007) and prox-1 has been applied to a study of equine normal tissue and lymphangioma (Staszyk *et al.*, 2005; Junginger *et al.*, 2010) and to investigate normal canine tissue (Saito *et al.*, 2006; Martin *et al.*, 2010). To the best of our knowledge, no studies of lymphatic vessels in CMTs have been performed.

Several studies have demonstrated clinical and molecular similarities between CMTs and human breast cancer (Khanna and Hunter, 2005; Porrello *et al.*, 2006; Lavalle *et al.*, 2009; Uva *et al.*, 2009; Klopfleisch *et al.*, 2011; Queiroga *et al.*, 2011a). Consequently, pet dogs with naturally occurring CMTs can serve as an animal model for the study of human breast cancer. In addition, the study of new human anti-cancer drugs (e.g. anti-angiogenic and anti-lymphangiogenic drugs) in dogs with spontaneously arising tumours provides an opportunity to acquire additional information related to the optimal dose, pharmacokinetics, pharmacodynamics and toxicity of the drug (Gordon and Khanna, 2010). Gaining more information on the presence of lymphatic vessels in CMTs should be the first step in establishing a comparative oncology model for anti-lymphangiogenic therapy.

The aim of the present study was to develop an immunohistochemical protocol for the identification of blood and lymphatic vessels in CMTs.

3.3. Materials and Methods

Samples

The study was conducted with 18 tissue samples. Ten of these were CMTs submitted after surgical excision to the Laboratory of Applied Veterinary Morphology of the University of Antwerp, Belgium. The tumours came from dogs of different breeds (four mixed breeds, two Labradors, one Jack Russell terrier, one Cairn terrier, one Barzoi and one Dachshund) and ages (5–15 years). Fresh samples were fixed in 4% neutral buffered formalin for 26 (±2) h and embedded in paraffin wax. Sections were stained by haematoxylin and eosin (HE). The CMTs were classified according to the criteria of the World Health Organization (Misdorp et al., 1999). Five benign tumours (three complex adenomas and two benign mixed tumours) and five malignant tumours (three solid carcinomas and two anaplastic carcinomas) were included.

Eight samples of healthy canine mammary tissue were collected immediately after death from bitches submitted for necropsy examination that did not show any mammary gland or reproductive tract abnormality. These dogs were 8–14 years of age and included two Shih Tzus, one American Staffordshire bull terrier, one Scottish terrier, one German shepherd dog, one Dachshund and two Miniature Schnauzers.

Immunohistochemistry

A variety of immunohistochemical protocols was tested with sections (5 µm) cut from the paraffin wax blocks for each primary antibody including polyclonal rabbit anti-human vWf (Dako, Glostrup, Denmark), monoclonal mouse anti-human CD31 (clone JC70A, Dako), monoclonal mouse anti-human CD34 (clone QBEnd 10, Dako), polyclonal rabbit anti-human prox-1 (RELIATech GmbH, Wolfenbüttel, Germany), polyclonal rabbit anti-human lyve-1 (RELIATech GmbH), monoclonal mouse anti-human podoplanin (RELIATech GmbH) and monoclonal mouse anti-human D2-40 (Dako). Three washes (5 min each) were performed with Dako wash buffer or 0.05 M triphosphate buffered saline (TBS; 60.6 g Tris, 90 g NaCl and 37 ml HCl, pH 7.4) between each step of the procedure. Combinations of several antigen retrieval methods (enzymatic or heat induced with microwave or high pressure cooker) and antigen retrieval buffers (trypsin; MP Biomedical, Illkirch, France), proteinase K (Dako), citrate buffer (pH 6.0) (Dako) and Tris–EDTA (pH 9.0; Dako) were tested. A variety of primary antibody dilutions and incubation times at different temperatures (60°C, 37°C, room temperature and 4°C) were applied. In addition, 'visualisation' of antibody binding was performed using an enzyme-conjugated secondary antibody (Envision™, Dako) and a labelled streptavidin–biotin (LSAB) method with different dilutions and incubation times.

Preliminary results indicated no binding of CD34 and podoplanin, so final immunohistochemical protocols were produced for vWf, CD31, prox-1 and lyve-1 (Table 3-1).

Reactions were 'visualised' using 3,3'-diaminobenzidine (DAB; Dako) and counterstaining with haematoxylin was performed. Positive controls included a section of canine haemangioma for vWf and CD31 and a section of canine lymph node for prox-1 and lyve-1. Blood and lymphatic vessels in normal mammary tissue and in non-tumour areas of the tumour tissue samples served as additional internal controls. For negative controls, the primary antibody was replaced with 0.05 M TBS containing 0.3% Triton X-100 (Sigma Aldrich, St Louis, Missouri, USA) and 1% bovine serum albumin (Sigma Aldrich).

Table 3-1 *Summary of the immunohistochemical protocols*

	vWf	CD31	Prox-1	Lyve-1
Target	Blood vessel endothelial cells	Blood vessel endothelial cells	Lymphatic vessel endothelial cells	Lymphatic vessel endothelial cells
Antigen retrieval	Enzymatic, 15 min 37°C Trypsin 0.05% (MP Biomedical)	HIER microwave, 20 min 90W Dako target retrieval solution Tris–EDTA buffer pH 9.0 (Dako)	HIER microwave, 15 min 90W Dako target retrieval solution citrate buffer pH 6.0 (Dako)	HIER microwave, 30 min 90W Dako target retrieval solution citrate buffer pH 6.0 (Dako)
Primary antibody	Polyclonal rabbit anti-human vWf (Dako) Dilution 1 in 500, incubation 4°C ON	Monoclonal mouse anti-human CD31, clone JC70A (Dako) Dilution 1 in 20, incubation 4°C ON	Polyclonal rabbit anti-human prox-1 (RELIATech) Dilution 1 in 100, incubation 60 min 37°C	Polyclonal rabbit anti-human lyve-1 (RELIATech) Dilution 1 in 25, incubation 24 h 4°C
Secondary antibody	EnVision + System-HRP labelled polymer anti-rabbit (Dako) 30 min RT	Polyclonal goat anti-mouse immunoglobulins/ biotinylated (Dako) 1 in 200, 30 min RT	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (Dako) 1 in 200, 30 min RT	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (Dako) 1 in 600, 120 min RT
Enzyme complex		Streptavidin–horseradish peroxidase, 1 in 200, 30 min RT	Streptavidin–horseradish peroxidase, 1 in 200, 30 min RT	Streptavidin–horseradish peroxidase 1 in 600, 120 min RT

W, Watts; ON, overnight; RT, room temperature; HIER, heat-induced epitope retrieval

Evaluation of Immunohistochemistry

All slides (18 samples labelled with five primary antibodies in duplicate, n = 180) were evaluated by two independent observers (N. Sleeckx and L. Van Brantegem) using an Olympus BX61 microscope (Olympus, Aartselaar, Belgium) equipped with

an Olympus DP50 digital camera connected to a computer system running the Olympus software program Analysis Pro™. The observers were unaware of the histopathological diagnosis ascribed to the tumour samples. Microvessel 'hotspots' were identified in HE-stained sections as areas containing numerous microvessels at low magnification (Vermeulen *et al.*, 2002). Two non-overlapping hotspots were chosen for the intratumoural (IT) and the peritumoural (PT) regions of the tissue. Three non-overlapping hotspots were selected in sections of normal mammary tissue. The same hotspots were identified in each serial section and the sections were evaluated at magnifications of ×200 and ×400. Six parameters were evaluated in each hotspot. When no immunoreactivity was observed in a large region of a mainly positively labelled slide, all hotspots within that region were recorded as 'missing' for the parameters 'all vessels labelled' and 'number of vessels labelled'.

All vessels labelled This parameter is a measure of the sensitivity of labelling and evaluated whether the blood or lymphatic vessels detected on the HE-stained sections were also labelled immunohistochemically.

Number of vessels labelled For this parameter, each labelled endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels, tumour cells, and connective tissue elements was considered as a single microvessel and enumerated (Weidner et al., 1991). The presence of a lumen, containing erythrocytes in the case of a blood vessel, was not necessary for a structure to be defined as a microvessel (Weidner et al., 1991; Restucci et al., 2000; Maiolino et al., 2001; Luong et al., 2006; Lavalle et al., 2009).

Only blood or lymphatic vessels labelled This parameter is another measure of the specificity of labelling and describes whether labelling of vessels other than those that were supposed to be detected by a particular antibody was present.

All endothelial cells labelled This parameter evaluated whether an immunohistochemical method labelled all the endothelial cells of a single vessel.

Intensity of labelling Intensity was scored on a four-point scale from low to high.

Unwanted background Non-specific background staining of tissues other than blood or lymphatic vessels was evaluated.

Statistical Analysis

Statistical analysis was performed using mixed model analysis with the numerical parameters analysed using a linear mixed model and the binary variables using a generalized linear mixed model. For all models, the variables (i.e. magnification [×200 or ×400], observer, tumour type [control, benign or malignant], hotspot location [control, IT or PT] and labelling type [vWf/CD31 or prox-1/lyve-1]) were defined as fixed factors. A hierarchical structure was entered for the random effects, with the random effect of hotspot nested within the random effect of sample.

For each of the parameters, the significance of the differences was tested for (1) the main effect of labelling type, which tests the presence of a significant difference between the two primary antibodies across all types of tissue (control, benign and malignant tumours) and locations (IT and PT), (2) the interaction between labelling type and tumour type, which tests the uniformity of the difference between the two labelling types across control tissues, benign and malignant tumours. In case of significance, the effect of labelling type was tested separately for control tissues, benign and malignant tumours, and (3) the interaction between labelling type and IT or PT location, which tests the uniformity of the difference between the two labelling types across IT hotspots, PT hotspots and control tissues. In case of significance, the effect of labelling type was tested separately for control tissues, IT and PT hotspots.

The other covariates (observer and magnification) were retained in the model when they were significant. All models were fitted using the lmer function in the lme4 package, in the statistical package R (www.r-project.org). Significance was tested using the likelihood ratio test. P <0.05 was considered significant.

3.4. Results

Blood vessels were labelled with antibodies specific for CD31 and vWf, and lymphatic vessels were labelled with antibodies specific for prox-1 and lyve-1 (Figure 3-1). Scores ascribed by the two observers were occasionally different, but there was little variance between duplicate measurements and no difference between the two magnifications. A summary of the data is presented in Tables 3-2 and 3-3.

All Vessels Labelled

For lymphatic vessels across all tumour types and controls, prox-1 performed significantly better in labelling all vessels (*P* <10E-16) (Figure. 3-2). The difference between prox-1 and lyve-1 remained the same regardless of the degree of malignancy of the tumour (*P* = 0.90). An interaction between labelling and tumour location was present (*P* = 0.007). For IT hotspots, the difference between prox-1 and lyve-1 was less than for control and PT hotspots.

For blood vessels, a first model with only main effects for labelling showed a significant difference between vWf and CD31 for the proportion of 'all vessels labelled' (*P* <2.5E-12). CD31 labelled more blood vessels across all tissue types and hotspot locations (Figure 3-2). No interaction with the type of tissue (*P* = 0.45) was present, which implied that the difference between vWf and CD31 did not differ between control samples, benign and malignant tumours. Across both labelling types, the sensitivity was higher in the control samples compared with the benign and malignant tumours (*P* = 8.1E-6, post-hoc test with Tukey correction). The differences between vWf and CD31 remained identical across IT and PT tissue and control samples (*P* = 0.88).

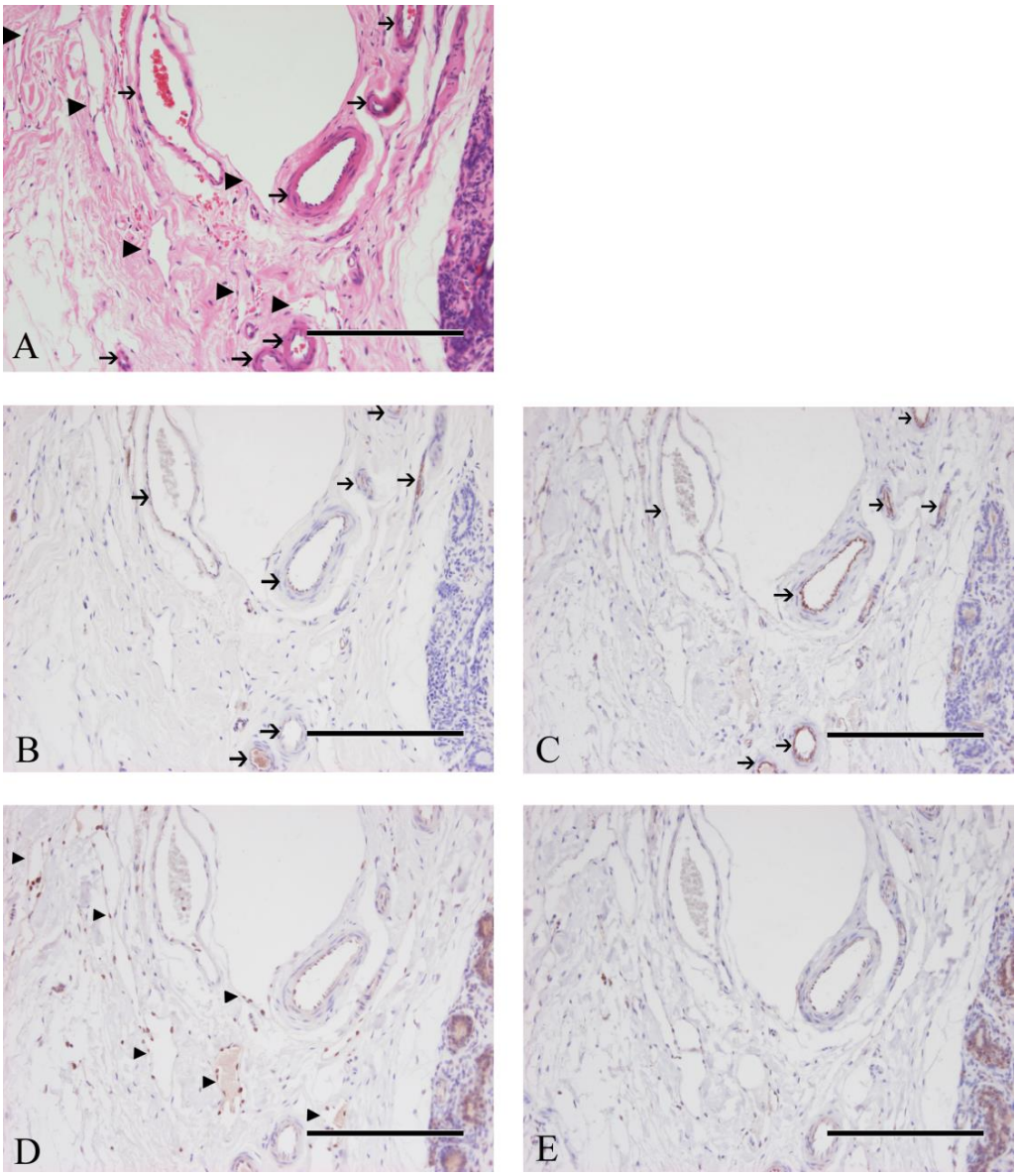

Figure 3-1 *Overview of the staining methods on serial slides with HE (A), blood vessel markers vWf (B) and CD31 (C), and lymphatic vessel markers Prox-1 (D) and Lyve-1 (E). Blood vessels are indicated with arrows, lymphatic vessel with arrowheads. Bars, 100 µm*

Table 3-2 *Best blood vessel marker for different parameters in different types of tumours and different tumour regions*

Parameter	Overall best	Control	Benign	Malignant	IT	PT
All vessels labelled = Sensitivity	CD31	CD31	CD31	CD31	CD31	CD31
Numbers of vessels labelled	CD31	CD31[ns]	CD31	CD31	CD31	CD31
Only BV stained = specificity	vWf	vWf	vWf	vWf	CD31	vWf
All endothelial cells labelled	CD31	CD31	CD31	CD31	CD31	CD31
Intensity	CD31	CD31	CD31	CD31	CD31	CD31
Unwanted background	CD31	CD31	vWf	CD31	CD31[ns]	CD31[ns]

[ns] = not significant, IT = intratumoural, PT = peritumoural, BV = blood vessel

Table 3-3 *Best lymphatic vessel marker for different parameters in different types of tumours and different tumour regions*

Parameter	Overall best	Control	Benign	Malignant	IT	PT
All vessels labelled = sensitivity	prox-1	prox-1	prox-1	prox-1	prox-1	prox-1
Numbers of vessels labelled	prox-1	prox-1[ns]	prox-1	prox-1	prox-1[ns]	prox-1
Only LV labelled = specificity	prox-1	prox-1	prox-1	prox-1	prox-1	prox-1
All endothelial cells labelled	prox-1	prox-1	prox-1	prox-1	prox-1	prox-1
Intensity	prox-1	prox-1	prox-1	prox-1	prox-1	prox-1
Unwanted background	prox-1	prox-1	prox-1[ns]	prox-1	lyve-1	prox-1

[ns] = not significant, IT = intratumoural, PT = peritumoural, LV = lymphatic vessel

Number of Vessels Labelled

For lymphatic vessels across all samples, prox-1 detected significantly more vessels compared with lyve-1 (P = 2.0E-4 for the main effect of labelling type). A significant interaction was observed between labelling and tumour type (P = 0.0041 for interaction). Within the group of benign tumours, the difference between prox-1 and lyve-1 was more pronounced (P = 4.6E-6) compared with the malignant tumours (P = 0.02), while no significant difference between prox-1 and lyve-1 was observed in the control tissues. Significant interaction between labelling type and location (P = 8.0E-8) was present. Splitting the dataset into control tissue, IT and PT hotspots revealed that the difference between prox-1 and lyve-1 was only significant for the PT hotspots (P = 1.1E-7, with prox-1 performing better), while no significant difference between prox-1 and lyve-1 for controls (P = 0.84) and IT samples (P = 0.78) was seen.

For blood vessel labelling, CD31 performed significantly better (P = 0.0001 for main effect of labelling) for both tumour samples and control tissues. A significant interaction between the labelling and the malignancy of the tumour was observed. While there was no significant difference between vWf and CD31 for control tissues, the CD31 labelling performed significantly better within the group of benign and malignant tumours (P-values of 0.006 and 0.0001, respectively).
The differences between vWf and CD31 were not uniform between hotspot locations and control tissues. A significant interaction between hotspot location and labelling type was observed (P = 5E-4). For control tissues, no significant difference (P = 0.44) was present between vWf and CD31. For both PT and IT hotspots, CD31 performed significantly better (P = 0.0026 and P = 3E-4, respectively).

Only Blood or Lymphatic Vessels Labelled

For lymphatic vessels, lyve-1 scored worse for all subgroups (malignancy, IT or PT) (P <2E-16) than prox-1 (Figure 3-2). The difference between prox-1 and lyve-1

varied slightly between control tissues, benign and malignant tumours, and also between control tissues, IT hotspots and PT hotspots.

For blood vessels, vWf showed a higher specificity (P = 0.002 for main effect of labelling). The difference was significantly more pronounced for the malignant tumours, compared with the control tissues and benign tumours (P = 0.03 for interaction between tumour type and labelling). A test for interaction between labelling and hotspot location showed that CD31 had a lower specificity in control tissues and PT hotspots, but not in IT hotspots (P = 0.04 for interaction between tumour location and staining) (Figure 3-2).

All Endothelial Cells Labelled

For lymphatic vessel labelling, lyve-1 performed worse than prox-1 across all tumour types (P <2E-16) and the difference between prox-1 and lyve-1 was not significantly different between control samples, benign tumours and malignant tumours (P = 0.34 for interaction tumour type-staining). A significant correlation with tumour location (P = 0.0003) was present, with the difference between prox-1 and lyve-1 being less pronounced in IT hotspots.

Blood vessel labelling was analogous to the 'all vessels labelled' parameter, and the results were identical. CD31 was significantly better than vWf (P = 0.004) in labelling all endothelial cells, across all tumour types, hotspot positions and control tissues. The malignancy of the tumour or the location of the hotspot (IT and PT) did not influence the difference between vWf and CD31, with the CD31 labelling systematically performing better.

Intensity of Labelling

For lymphatic vessels, lyve-1 had a lower labelling intensity compared with prox-1 across the different sample types (P <2E10-16). The difference between prox-1 and lyve-1 was uniform across control tissues, benign tumours and malignant tumours. The largest difference between prox-1 and lyve-1 was observed for PT hotspots (P <2E10-16).

For blood vessels, CD31 showed a higher intensity of labelling across the different sample types. For benign tumours, the difference was the largest (*P* = 2E10-16). CD31 had a higher intensity of labelling than vWf for control tissues, IT hotspots and PT hotspots. For IT hotspots, the difference between vWf and CD31 was the largest (*P* = 0.0027 for interaction between location and labelling). For malignant tumours, the difference between vWf and CD31 was less pronounced compared with control tissues and benign tumours (*P* = 0.005 for interaction between labelling and tumour type).

Unwanted Background

For lymphatic vessels, the model with main effects showed more background with lyve-1 (*P* = 0.0005), but an interaction with tumour type (*P* = 1.8E-4) was observed. While no significant difference in background was observed for malignant tumours (*P* = 0.31), prox-1 showed significantly less background for controls and benign tumours (*P* = 1.8E-4 and 0.0014, respectively).

A significant interaction was noticed between labelling and hotspot location (IT or PT) (*P* = 4.5E-6). Lyve-1 showed more background in control tissues and PT hotspots, and less background for IT hotspots. Within the group of PT and IT hotspots, the difference between prox-1 and lyve-1 was not significant (*P* = 0.31 and *P* = 0.07, respectively).

For blood vessels, the model with only main effects showed that CD31 produces less background, but the difference was only marginally significant (*P* = 0.051). When assessing the malignancy of the tumour, an interaction between tumour type and labelling was observed. CD31 gave less background for control tissues and malignant tumours, while for benign tumours (*P* <2E-16 for interaction between labelling and tumour type) vWf showed less background. CD31 showed consistently less background than vWf across control tissues, PT hotspots and IT hotspots, but this difference was not significant (*P* = 0.051).

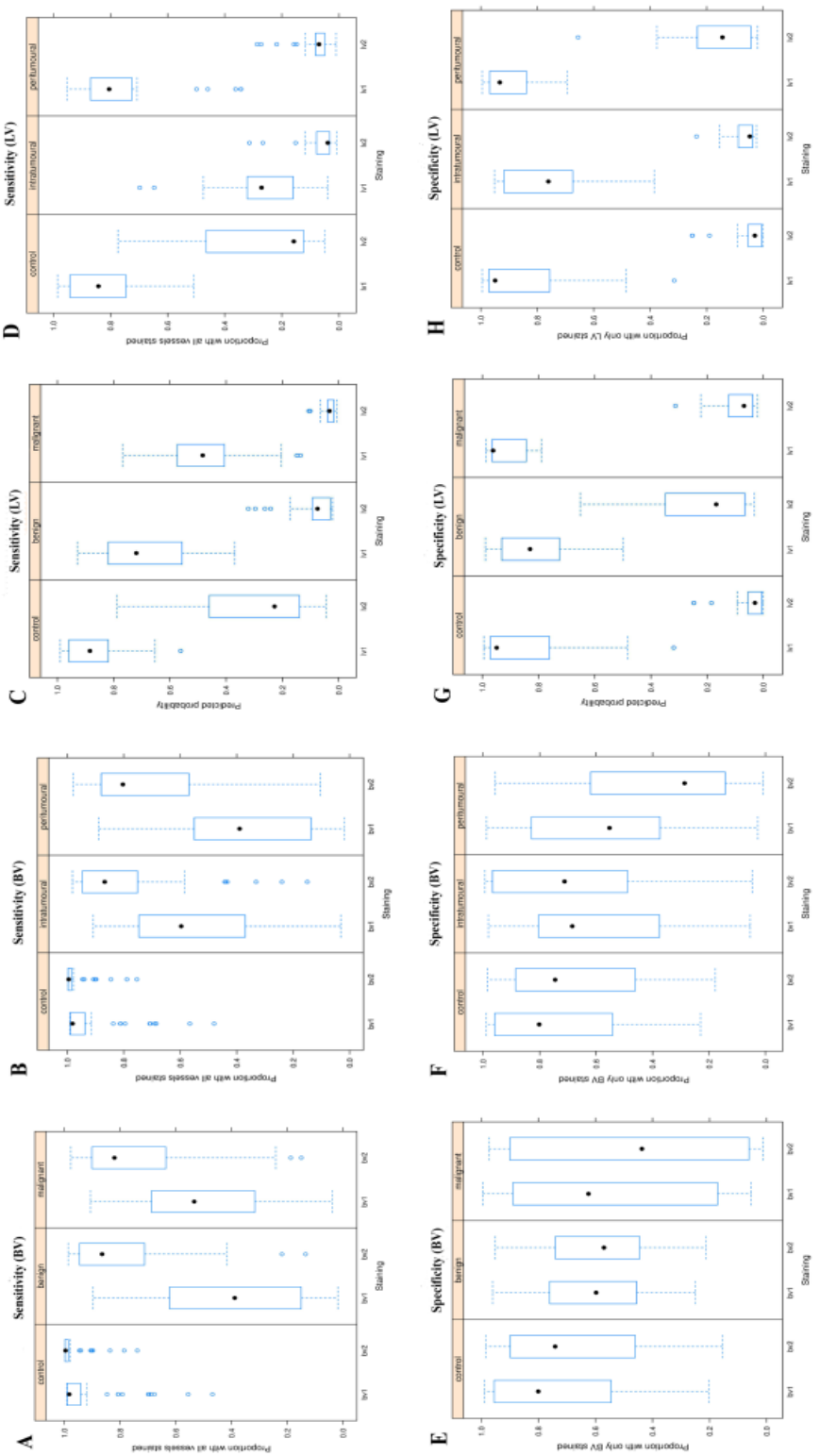

Figure 3-2 *Boxplots showing the predicted sensitivity of blood (A and B) and lymphatic vessels (C and D), and the predicted specificity of blood (E and F) and lymphatic vessels (G and H). BV, blood vessels; LV, lymphatic vessels*

3.5. Discussion

Dogs with spontaneously arising CMTs are considered a promising animal model of human breast cancer (Khanna and Hunter, 2005; Porrello *et al.*, 2006; Lavalle *et al.*, 2009; Uva *et al.*, 2009; Klopfleisch *et al.*, 2011; Queiroga *et al.*, 2011b) and may prove useful for trials of anti-angiogenic and anti-lymphangiogenic therapies. In order to be used in such comparative oncology studies, lymphatic vessels in dogs with CMTs must be identified unambiguously. The ideal immunohistochemical marker for this purpose should have a high sensitivity and specificity, show low background staining, detect canine lymphatic vessels and detect all vessel segments during disease progress (Van der Auwera *et al.*, 2006; Baluk and McDonald, 2008).

As no canine lymphatic vessel markers are available, antibodies directed towards human lymphatic endothelial cells (prox-1, lyve-1, podoplanin and D2-40) were investigated. Although vascular endothelial growth factor receptor-3 (VEGFR-3), the receptor for VEGF-C, has also been described as a marker of lymphatic vessels (Saito *et al.*, 2006; Van der Auwera *et al.*, 2006; Sarli *et al.*, 2007; Baluk and McDonald, 2008), this marker was not tested since VEGFR-3 only labels recently induced lymphatics and can also be expressed by epithelial tumour cells, macrophages and blood vessels (Reis-Filho and Schmitt, 2003; Al-Rawi *et al.*, 2005; Van der Auwera *et al.*, 2006; Sarli *et al.*, 2007; Baluk and McDonald, 2008; Sleeman *et al.*, 2009; Holopainen *et al.*, 2011). Only prox-1 and lyve-1 showed immunoreactivity with canine lymphatic vessels. Although podoplanin and D2-40 did not label lymphatic vessels in formalin-fixed and paraffin wax-embedded tissues, it is possible that these reagents may still be applicable to sections of fresh-frozen tissue.

Further comparison of prox-1 and lyve-1 showed prox-1 to be the most suitable lymphatic vessel marker for canine normal and neoplastic mammary tissue. The IT regions scored poorly compared with the normal (control tissue) and PT regions

for sensitivity and intensity of labelling for both prox-1 and lyve-1. Studies of human breast cancer have shown that the expression of some markers is increased or decreased in tumour vasculature (Van der Auwera *et al.*, 2004; Baluk and McDonald, 2008); for example in some studies there is decreased or even absent expression of lyve-1 within tumour tissue (Williams *et al.*, 2003; Vleugel *et al.*, 2004; Van der Auwera *et al.*, 2005).

Lymphatic vessel markers must be applied in parallel with blood vessel markers in order to discriminate between the two vessel types (Van den Eynden *et al.*, 2006). The application of blood vessel markers (vWf, CD31 and CD34) to canine tissues has been described, with vWf and CD31 used most often. However, a detailed comparison of these markers in CMTs has not yet been described. There are contradictory reports on the immunoreactivity of anti-CD34 antibodies with canine tissue. Wolfesberger et al. (2008) state that anti-CD34 antibodies do not label vessels in formalin-fixed and paraffin wax-embedded sections of canine tissue, while Clemente et al. (2010) describe labelling of some endothelial cells in canine non-inflammatory carcinomas. As CD34 did not show any immunoreactivity with canine normal and neoplastic mammary tissue in the present study, only vWf and CD31 were further analysed. CD31 was found to be the most suitable blood vessel marker, since it scored best for almost all parameters, except for specificity. As the blood vessel marker needs to be used in parallel with the specific lymphatic vessel marker prox-1, the lower specificity of CD31 will not cause any interpretation problems.

Earlier studies confirmed the low sensitivity of vWf and described the possible absence of vWf reactivity in small blood vessels and capillaries in human (Vermeulen *et al.*, 1996; Uzzan *et al.*, 2004) and canine tissue (Tonar *et al.*, 2008; Wolfesberger *et al.*, 2008). Similar to the lymphatic vessel markers, the sensitivity, specificity and intensity of labelling of the blood vessel markers were higher in control tissues compared with neoplastic tissues. Our findings contrast with the

comparison of vWf and CD31 performed by Luong et al. (2006), where vWf was reported to be the best marker.

For both the blood and lymphatic vessel labelling methods, some background staining of inflammatory cells (in particular macrophages and plasma cells), normal epithelium and neoplastic epithelial cells in benign and malignant tumours was observed. It is known that these cell types cross react with endothelial markers (Vermeulen *et al.*, 1996; Uzzan *et al.*, 2004; Luong *et al.*, 2006; Van der Auwera *et al.*, 2006; Baluk and McDonald, 2008; Martin *et al.*, 2010). However, CD31 and prox-1 appeared to have an overall lower background staining compared with vWf and lyve-1. It has been suggested that inflammation and angiogenic or lymphangiogenic factors can play a role in this phenomenon (Van der Auwera *et al.*, 2006; Johnson *et al.*, 2007; Baluk and McDonald, 2008). As the slides were counterstained with haematoxylin in the present study, differentiation between background staining and stained vessels was possible in most, if not all, cases.

In conclusion, the present methodology enables labelling of lymphatic and blood vessels and clear differentiation of lymphatic vessels from blood vessels in CMTs. Prox-1 was the most sensitive and specific lymphatic vessel marker, since it reproducibly labelled lymphatic vessels with limited background staining. In combination with CD31, the best blood vessel marker, a reliable means of evaluating angiogenesis and lymphangiogenesis was established for normal canine mammary tissue and CMTs. This method creates opportunities for examining the role of lymphangiogenesis in canine mammary neoplasia and may provide important prognostic information. The ability to identify lymphatic vessels in CMTs may be the first step in establishing an animal model for human breast cancer lymphangiogenesis.

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CHAPTER 4: Lymphangiogenesis in canine mammary tumours: a morphometric and prognostic study

Based on

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4.1. Abstract

Canine mammary tumours (CMTs) are the most common neoplasms in intact female dogs and represent a promising model for human breast cancer. Unfortunately, little is known on the presence and prognostic value of lymphangiogenesis in CMTs. The aims of the present study were to analyse a variety of lymphatic vessel characteristics in CMTs, to evaluate their prognostic significance and to compare these results with human breast cancer studies. Fifty-six benign CMTs, 55 malignant CMTs and 13 control samples of normal canine mammary gland tissue were studied. Serial immunohistochemical labelling with the lymph vessel marker prox-1 and the proliferation marker Ki67 was performed. In intratumoural (IT) and peritumoural (PT) regions, the lymphatic vessel density (LVD), mean lymphatic vessel perimeter (LVP) and relative area occupied by lymphatic vessels (LVA) were analysed. In addition, lymphatic endothelial cell proliferation (LECP) together with tumour cell proliferation (TCP) were measured. Lymphatic vessels were identified in IT and PT regions and ongoing lymphangiogenesis was present in both regions. The IT lymphatic vessels were smaller, less numerous and occupied a smaller relative area compared to those of the PT region. Although no differences in lymphatic vessel parameters were observed between benign and malignant tumours, control tissue differed significantly from neoplastic tissue. None of the lymphatic vessel parameters showed a prognostic value, except for LECP in PT regions of benign tumours. In conclusion, most findings are in accordance with results in human breast cancer studies, which supports the use of dogs with spontaneously occurring CMTs as an animal model in comparative oncology trials.

4.2. Introduction

Mammary tumours are the most common tumours in women and bitches. More than 40% of all tumours in intact female dogs are canine mammary tumours (CMTs) and approximately 50% are malignant (MacEwen and Withrow, 1996; Sleeckx *et al.*, 2011). Almost half of the affected dogs die or are euthanized within

1 year of surgery because of tumour recurrence or metastasis (Graham and Myers, 1999). Survival can vary significantly depending on different tumour and host characteristics, yet the prognosis of CMTs remains difficult to predict (Perez Alenza *et al.*, 1997; Sleeckx *et al.*, 2011; Santos *et al.*, 2013). Therefore, there is a need to define additional features that can predict the biological behaviour of CMTs.

Tumour growth induces both angiogenesis and lymphangiogenesis. The formation of these new blood and lymphatic vessels originating from the pre-existing vascular network is essential for tumour growth, invasion and metastasis (Folkman, 1986; Fox *et al.*, 1996; Saaristo *et al.*, 2000; Al-Rawi *et al.*, 2005; Sleeman and Thiele, 2009; Holopainen *et al.*, 2011). In human medicine, angiogenesis has been studied in great detail in breast cancer (Weidner *et al.*, 1991; Uzzan *et al.*, 2004; Van der Auwera *et al.*, 2004). During the last two decades, some research on this topic has also been performed in different canine tumour types including CMTs (Griffey *et al.*, 1998; Graham and Myers, 1999; Restucci *et al.*, 2000; Luong *et al.*, 2006; Lavalle *et al.*, 2009; Im *et al.*, 2011). However, to our knowledge, information on lymphangiogenesis in and around CMTs and its association with clinical features is still lacking. A previous study evaluated immunohistochemical blood and lymphatic vessel markers in normal and tumoural canine mammary tissue. The homeodomain protein prospero-related homeobox I (prox-1) was shown to be the most sensitive and specific lymphatic marker throughout all tissue types (Sleeckx *et al.*, 2013). This lymphatic marker can be used to investigate lymphatic vessels in CMTs. Active lymphangiogenesis can be assessed by evaluating prox-1 expression in combination with that of the proliferation marker Ki67 and CD31 labelling allows discrimination between lymphatic and blood vessels (Van den Eynden *et al.*, 2006).

As CMTs show similarities to human breast cancer (Khanna and Hunter, 2005; Porrello *et al.*, 2006; Lavalle *et al.*, 2009; Uva *et al.*, 2009; Klopfleisch *et al.*, 2011; Queiroga *et al.*, 2011; Casteleyn *et al.*, 2013), pet dogs with naturally occurring CMTs could be used as an animal model in comparative oncology trials. They could provide additional information regarding anti-angiogenic and anti-lymphangiogenic therapeutics, which have gained great interest in human breast cancer studies (Ferrara *et al.*, 2005; Thiele and Sleeman, 2006; Stacker and Achen, 2008; Potente *et al.*, 2011; Witte *et al.*, 2011). Acquiring more information about lymphatic vessels in CMTs is required to establish a comparative oncology model for anti-lymphangiogenic therapy. Therefore, the aims of this study were (a) to thoroughly investigate lymphatic vessel characteristics in intratumoural (IT) and peritumoural (PT) regions, both in benign and malignant CMTs, (b) to assess the proliferative status of tumour associated lymphatics as an indicator of ongoing lymphangiogenesis and (c) to examine the prognostic value of these lymphatic vessel characteristics.

4.3. Materials and Methods

Samples

One hundred and twenty four mammary gland samples were collected. Samples of healthy canine mammary gland (*n* = 13) were collected during necropsy examination of bitches with normal non-neoplastic mammary glands. These dogs ranged in age from 6 to 16 years (mean age 10.75 years) and different breeds were represented. The CMTs (*n* = 111) were surgically removed from female dogs with a mean age of 10 years (age range from 5 to 17 years) and were submitted to the Laboratory of Applied Veterinary Morphology of the University of Antwerp, Belgium. The CMTs were classified according to the World Health Organization's diagnostic criteria (Misdorp *et al.*, 1999). Grading of the malignant tumours was performed according to the Elston and Ellis method adapted to CMTs (Clemente *et al.*, 2010; Pena *et al.*, 2013). Clinical follow-up data (i.e. recurrence, metastases

and survival) were obtained from the case records, or from client and veterinarian follow-up for a minimum of 12 months.

Immunohistochemistry

Samples were fixed in 4% neutral buffered formaldehyde, processed routinely and embedded in paraffin wax. Serial sections were stained by haematoxylin and eosin (HE) and labelled immunohistochemically for expression of prox-1 (RELIATech, Wolfenbüttel, Germany), Ki67 (Dako, Gostrup, Denmark) or CD31 antibody (Dako). A summary of the immunohistochemistry (IHC) protocols can be found in Table 4-1. Three washes with Dako wash buffer were performed between each step of the procedure. Reactions were 'visualised' using 3,3'-diaminobenzidine (DAB; Dako) and counterstaining with haematoxylin was performed. Positive controls included a section of canine haemangioma and lymph node for CD31 and prox-1, respectively. Blood and lymphatic vessels in normal mammary tissue and in non-tumour areas of the tumour tissue samples served as additional internal controls. Epidermis on the sections was used as internal positive control for Ki67 because of the physiological presence of proliferating keratinocytes in the stratum basale. For negative controls, the primary antibody was replaced with 0.05M TBS containing 0.3% Triton X-100 (Sigma Aldrich, St Louis, Missouri, USA) and 1% bovine serum albumin (Sigma Aldrich).

Table 4-1 *Summary of the immunohistochemical protocols*

	CD31	***Ki67***	***Prox-1***
Target	Blood vessel endothelial cells	Proliferating cells	Lymphatic vessel endothelial cells
Antigen Retrieval	HIER microwave, 20 min 90W Dako target retrieval solution Tris-EDTA buffer pH 9.0 (Dako)	HIER microwave, 15 min 90W Dako target retrieval solution citrate buffer pH 6.0 (Dako)	HIER microwave, 15 min 90W Dako target retrieval solution citrate buffer pH 6.0 (Dako)
Primary Antibody	Monoclonal mouse anti-human CD31, clone JC70A (Dako)	Monoclonal mouse anti-human Ki67, clone MIB-1 (Dako)	Polyclonal rabbit anti-human prox-1 (RELIATech)
	Dilution 1 in 20 incubation 4°C ON	Dilution 1 in 25 incubation 60 min 37°C	Dilution 1 in 100 Incubation 60 min 37°C
Secondary antibody	Polyclonal goat anti-mouse immunoglobulins/ biotinylated (Dako) 1 in 200, 30 min RT	Polyclonal goat anti-mouse immunoglobins/ biotinylated (Dako) 1 in 200, 60 min RT	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (Dako) 1 in 200, 30 min RT
Enzyme complex	Streptavidine-horseradish peroxidase (Dako), 1 in 200, 30 min RT	Streptavidine-horseradish peroxidase (Dako), 1 in 200, 60 min RT	Streptavidine-horseradish peroxidase (Dako), 1 in 200, 30 min RT

W, Watts; ON, overnight; RT, room temperature; HIER, heat-induced antigen retrieval.

Assessment of morphological characteristics

All slides (4 x 124, *n* = 496) were evaluated using an Olympus BX61 microscope (Olympus, Aartselaar, Belgium) equipped with an Olympus DP50 digital camera connected to a computer system running the Olympus software program Analysis Pro™. Image analysis was performed without knowledge of clinicopathological details. Microvessel 'hotspots' were identified in HE-stained sections as areas giving the impression at low magnification (x40 and x100) of containing numerous microvessels (Vermeulen *et al.*, 2002). Two non-overlapping hotspots were chosen for intratumoural (IT) and peritumoural (PT) regions of the tissue. Three non-overlapping hotspots were selected in sections of normal mammary tissue. Digital images of these hotspots were taken at magnification x200. In each hotspot, the number of lymphatic vessels per mm² (lymphatic vessel density =

LVD), the total and average lymphatic vessel perimeter (LVP) and the total and relative area occupied by lymphatic vessels (LVA) were analysed. Evaluation of the microvessels was performed according to the method of Weidner (1991). In addition, the percentage of proliferating lymphatic endothelial cells (LECP) together with the tumour cell proliferation (TCP) were calculated. The LECP was determined in each hotspot as the number of lymphatic endothelial cells with Ki67-positive stained nuclei per 100 lymphatic endothelial cells. The same method was applied for the calculation of the TCP.

Statistical analysis

Evaluation of lymphatic vessel density, lymphatic vessel area, lymphatic vessel perimeter, lymphatic endothelial cell proliferation and tumour cell proliferation

Statistical analyses were performed using the statistical package R (www. R-project.org). Linear mixed models were fitted in the lmer function in the lme4 package in R. For TCP, with only one measurement per tumour, a one-way ANOVA was fitted with proliferation (number of Ki67-positive tumour cells per 100 tumour cells) as outcome variable and malignancy as explanatory variable. All other variables were analysed by fitting linear mixed models that included a random intercept term for tumour number, to account for the dependency between observations within the same tumour. As fixed effects, malignancy and the position within the tumour (IT or PT) were entered, as well as the interaction between them. In this model, we first tested for interaction between benign and malignant tumours. After removing the non-significant interaction term, the model was refitted with only main effects for IT/PT and malignancy and the significance of these two main effects was calculated. Significance of the interaction term and the main effects were tested using a likelihood ratio test (Agresti, 2002). Additional tests to separately compare the controls with the benign and malignant tumours were performed using the same linear mixed model framework. To compare the LECP values in control tissue versus IT and PT regions, both in malignant and benign tumour samples, pairwise Mann-Whitney

tests were performed. To test whether the Ki67 based tumour proliferation was different between malignant and benign tumours, a one-way ANOVA was performed. For IT and PT tissues separately, the difference in the presence of lymphatic vessels and LECP in benign and malignant tumours was tested using a χ^2 test.

Log-transformed values were used in all analyses, except for the proliferation parameters. A *P*-value of < 0.05 was considered statistically significant.

Association of lymphatic vessel density, lymphatic vessel area, lymphatic vessel perimeter, lymphatic endothelial cell proliferation and tumour cell proliferation with survival time

Survival analysis were carried out using the software package R, with the coxph function in the survival package. Follow-up data were expressed as disease free survival (DFS) time, defined as the interval between surgery and evidence of local recurrence and/or metastases, and as overall survival (OS) time, defined as the interval between surgical treatment and death due to the tumour. In case no recurrence or metastasis (for DFS) or no death due to tumour (for OS) had been observed by the end of the study period and/or in case the patient was lost to follow-up or a different event making a further follow-up impossible (including death from an unrelated illness) (Webster *et al.*, 2011), the last time point without event was recorded and an individual was considered "censored". To find out which parameters had the strongest association with surviving time, taking into account the censoring, a Cox proportional hazards regression model was fitted (Cox, 1972). The different lymphatic vessel parameters were entered as covariates. The predictive accuracy of the model was evaluated using the concordance between the observed order (of the event) and the predicted order in the model. The closer the value is to 1, the better the parameter predicts survival (Gönen and Heller, 2005).

4.4. Results

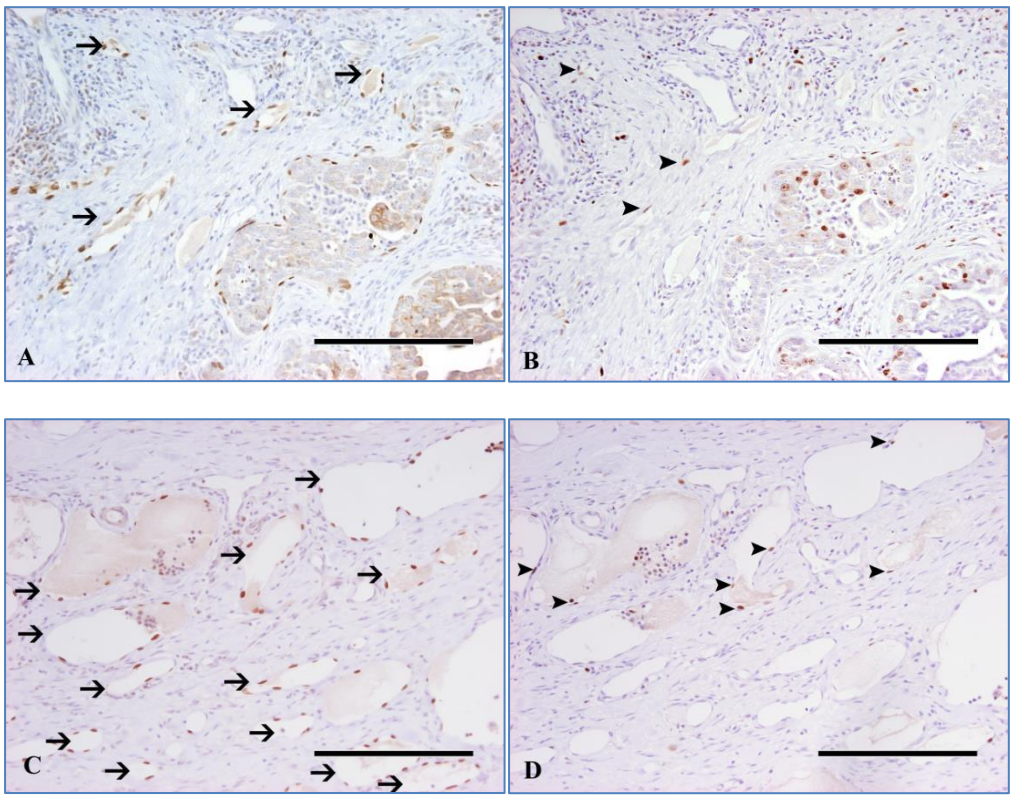

Figure 4-1 *Intratumoural (A and B) and peritumoural (C and D) region of a solid carcinoma with focal tubular differentiation. (A and C) prox-1 immunohistochemistry (IHC), (B and D) Ki67 IHC. Lymph vessels are indicated with arrows, proliferating lymphatic endothelial cells with arrowheads. Scale bar, 200 µm.*

Different histological types of CMTs were present. Of the 111 tumoural specimens, 56 (50.5%) were benign (simple adenoma (*n* = 14), complex adenoma (*n* = 25), benign mixed tumour *(n* = 17)) and 55 (49.5%) were malignant (tubulopapillary carcinoma (*n* = 10), solid carcinoma (*n* = 36), anaplastic carcinoma (*n* = 5), complex carcinoma (*n* = 3), carcinosarcoma (*n* =1)). Grade II tumours were overrepresented (*n* = 29), followed by grade I (*n* = 12) and grade III (*n* = 14). About a fifth (23/111) of the dogs was lost to follow-up directly after surgery and therefore, could not be included in survival analysis.

Evaluation of lymphatic vessel density, lymphatic vessel area, lymphatic vessel perimeter, lymphatic endothelial cell proliferation and tumour cell proliferation

IT lymphatic vessels were present in 51.9% of benign tumours and in 48.2% of malignant tumours (P = 0.59). PT lymphatic vessels were present in 94.5% of benign CMTs and in 93% of malignant CMTs (P = 0.84). Figure 4-1 shows IT and PT lymphatic vessels and proliferating lymphatic endothelial cells.

No significant difference was observed between control tissue and benign or malignant tumours for the number of lymphatic vessels. Yet, significantly higher relative LVA and mean LVP in malignant tumours were found compared to control mammary tissue. The PT lymphatic vessels were more numerous, occupied a larger area and had a larger perimeter than their IT counterparts (P < 0.01). As the interaction between malignancy and IT/PT was nowhere significant, this difference was identical in benign and malignant tumours. None of the lymphatic vessel parameters showed significant difference between benign and malignant tumoural tissue. PT and IT lymphatic vessels in malignant tumours shared their morphology with those in benign tumours.

To compare the current results with data reported previously from studies of human breast cancer, several additional comparisons were made between control tissue and the different regions of benign and malignant tissue. A significantly lower number of lymphatic vessels and lower total, mean and median area was found in both benign and malignant IT regions compared to control tissue. In addition, a significantly higher relative area, total perimeter and mean perimeter could be found in both benign and malignant PT regions versus controls.

Tables 4-2 and 4-3 show median values of the different parameters with corresponding *P*-values.

Proliferating lymphatic endothelial cells were seen in benign and malignant CMTs. IT LECP was present in 19.2% of benign tumours and in 12.3% of malignant

tumours (*P* = 0.299). PT LECP was present in 23.8% of benign tumours and in 42.5% of malignant tumours (*P* = 0.009). When considering the LECP values, a significant difference was found between the LECP in controls and malignant tumours. In addition, PT LECP was significantly higher compared to IT LECP. As no significant interaction between the malignancy and location was present, the difference between IT and PT was the same across benign and malignant tumours. LECP values differed significantly between the control samples and the different tumour regions, both in benign and malignant tumours. The least significant differences were found between controls and the malignant IT region. On correction for multiple testing, this pairwise comparison was no longer significant. All other comparison between control and tumour tissue remained significant on multiple testing correction. A summary of the median LECP values and *P*-values can be found in Tables 4-2 and 4-3.

Significant higher tumour cell proliferation was present in malignant tumours compared to benign tumours (*P* = 0.0057). On average the Ki67-value was 8.64 units higher in malignant tumours compared to benign tumours.

Association of lymphatic vessel density, lymphatic vessel area, lymphatic vessel perimeter, lymphatic endothelial cell proliferation and tumour cell proliferation with survival time

None of the lymphatic vessel parameters gave a consistently reliable prediction across all types of tumours and none of them was significantly associated with DFS or OS on Cox proportional hazards analysis. However, only sparse data were available to perform this analysis, as many values were either missing or equal to zero. Therefore, the algorithm for the Cox proportional hazard analysis failed to converge, especially in the group of benign tumours.

Only in PT benign tumours, LECP did have some predictive value, both for DFS and OS (concordance of 0.847 and 0.815, respectively). No association was found between the TCP and OS or DFS.

Table 4-2 *Lymphatic vessel related parameters in normal mammary tissue, benign CMTs and malignant CMTs*

Parameter	***Control***	***Benign Tumours***	***Malignant Tumours***	**P_1** ***value***	**P_2** ***value***
LVD/HPF	5	5	4	0.75	0.33
LVD/mm²	17.24	17.24	13.79	0.42	0.15
Total LVA (µm²)	3462.27	5240.28	5616.67	0.40	0.39
Mean LVA (µm²)	638.99	695.36	1153.34	0.44	0.62
Median LVA (µm²)	480.56	481.67	606.25	0.28	0.48
Relative LVA (%)	1.18	1.79	1.91	0.08	**0.03**
Total LVP (µm)	1444.53	1915.08	1464.45	0.14	0.18
Mean LVP (µm)	233.06	239.66	283.06	0.12	**0.01**
Median LVP (µm)	228.66	205.86	246.55	0.63	0.20
LECP (%)	0	0	0	0.06	**0.01**

Values in table are presented as median, *P*-values in bold are considered significant (< 0.05) LVD, lymphatic vessel density; HPF, high power field 400x magnification; LVA, lymphatic vessel area; LVP, lymphatic vessel perimeter; LECP, lymphatic endothelial cell proliferation; control = normal mammary gland tissue. P_1 value represents the comparison of control tissue versus benign tumours, P_2 value represents the comparison of control tissue versus malignant tumours.

Table 4-3 *Lymphatic vessel related parameters in normal mammary tissue and intra- and peritumoural areas in benign and malignant CMTs*

Parameter	*Control*	*Benign CMTs*		*Malignant CMTs*		P_1 *value*	P_2 *value*	P_3 *value*	P_4 *value*	P_5 *value*	P_6 *value*
		IT	**PT**	**IT**	**PT**						
LVD/HPF	5	1	7	0	7	0.27	**7.04E-19**	**0.009**	0.06	**8.10E-04**	0.17
LVD/mm²	17.24	3.45	24.14	0	24.14	0.31	**2.22E-20**	**0.001**	0.13	**7.00E-05**	0.28
Total LVA (µm²)	3462.27	315.37	12070.36	0	18453.730	0.78	**1.01E-20**	**5.20E-04**	0.10	**2.70E-04**	0.06
Mean LVA (µm²)	638.99	155.58	1414.10	0	2742.39	0.39	**2.07E-18**	**0.001**	0.12	**0.002**	**0.039**
Median LVA (µm²)	480.56	125.78	706.63	0	1145.84	0.18	**3.10E-18**	**6.90E-04**	0.26	**8.30E-04**	0.55
Relative LVA (%)	1.18	0.11	4.11	0	6.29	0.45	**3.76E-14**	0.95	**5.7E-04**	0.87	**2.00E-05**
Total LVP (µm)	1444.53	1300.82	2097.60	1063.76	2126.77	0.85	**7.02E-08**	0.60	**0.02**	0.44	**0.026**
Mean LVP (µm)	233.06	213.33	249.85	255.26	297.91	0.19	**0.005**	0.89	**0.03**	0.19	**0.006**
Median LVP (µm)	228.66	186.70	224.85	210.30	250.37	0.23	**0.009**	0.49	0.29	0.81	0.09
LECP	0	0	0	0	0	0.42	**0.018**	**0.006**	**0.001**	**0.030**	**5.90E-06**

Values in table are presented as median, *P*-values in bold are considered significant (< 0.05). IT, intratumoural; PT, peritumoural; LVD, lymphatic vessel density; HPF, high power field 400x magnification; LVA, lymphatic vessel area; LVP, lymphatic vessel perimeter; LECP, lymphatic endothelial cell proliferation; control = normal mammary gland tissue. P_1 value represents the comparison of benign versus malignant tumours (both for IT and PT regions as interaction was not significant), P_2 value represents the comparison of IT regions versus PT region (both for benign and malignant tumours as interaction was not significant), P_3 value represents the comparison of control versus benign intratumoural regions, P_4 value represents the comparison of control versus benign peritumoural regions, P_5 value represents the comparison of control versus malignant intratumoural regions, P_6 value represents the comparison of control versus malignant peritumoural regions.

4.5. Discussion

Although lymphatic metastasis is considered as the main route for metastasis of CMTs, the study of lymphatic vessels and in particular lymphangiogenesis, remains an under-researched area in veterinary medicine. Even in human breast cancer, tumoural lymphangiogenesis research has only gained great interest during the last two decades. Several studies have described the induction of new lymphatic vessels in and around the tumour and acknowledge the prognostic value of lymphatic vessels for the patient's survival and tumour metastasis(El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009; Mohammed *et al.*, 2009; Ran *et al.*, 2010; Kandemir *et al.*, 2012). As dogs with spontaneously arising CMTs are considered promising animal models of human breast cancer (Khanna and Hunter, 2005; Porrello *et al.*, 2006; Lavalle *et al.*, 2009; Uva *et al.*, 2009; Klopfleisch *et al.*, 2011; Queiroga *et al.*, 2011; Pinho *et al.*, 2012), analysis of lymphangiogenesis in CMTs is required in order to establish a comparative oncology model for anti-lymphangiogenesis therapy.

A recent study showed that lymphatic vessels in dogs can be identified with the lymphatic marker prox-1 (Sleeckx *et al.*, 2013). In combination with CD31, the best blood vessel marker as shown in the previous study, differentiation from blood vessels and evaluation of the lymphatic vessels in CMTs is now possible. Although the distribution of lymphatic vessels in so-called microvessel hotspots is questioned (Van der Auwera *et al.*, 2006; Ran *et al.*, 2010; Kandemir *et al.*, 2012), most analysis in human breast cancers are performed in hotspots (Agarwal *et al.*, 2005; Van der Auwera *et al.*, 2005; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009; Mohammed *et al.*, 2009; Kandemir *et al.*, 2012). Therefore, for comparison reasons, the same method was used in this study.

IT lymphatic vessels were found in about half of the tumours and PT lymphatic vessels in more than 90% of the tumours. These results correspond to human breast cancer studies, which show IT lymphatic vessels to be present in 0% to

more than 80% and PT lymphatic vessels to be present in 36% to 97% of the analysed breast samples (Williams *et al.*, 2003; Vleugel *et al.*, 2004; Agarwal *et al.*, 2005; Van der Auwera *et al.*, 2005; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009; Mohammed *et al.*, 2009; Kandemir *et al.*, 2012). Most studies that failed to detect IT lymphatic vessels used the lyve-1 antibody instead of the prox-1 or podoplanin/D2-40 antibodies (Williams *et al.*, 2003; Vleugel *et al.*, 2004; Agarwal *et al.*, 2005; Van der Auwera *et al.*, 2005; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009; Mohammed *et al.*, 2009; Kandemir *et al.*, 2012). The highest percentage of IT lymphatics was accomplished with the D2-40 lymphatic marker, which shows no immunoreactivity in CMTs (Sleeckx *et al.*, 2013). The diversity in results for lymphatic vessel detection can possibly be explained by the heterogeneous nature of the tumour types examined, by the variable patient population and by the variety in techniques used (El-Gohary *et al.*, 2008; Kandemir *et al.*, 2012).

In the benign and malignant CMTs in this study, the IT lymphatic vessels were smaller, less numerous and occupied a smaller relative area compared to the PT lymphatics. This is in accordance with the results of Van der Auwera *et al* (2005) in breast carcinomas. The increased LVD in the PT tissue compared to the IT tissue was also found in the majority of human breast cancer studies (Van der Auwera *et al.*, 2005; Ito *et al.*, 2007; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009; Mohammed *et al.*, 2009; Kandemir *et al.*, 2012). Most of these studies only investigated breast carcinomas, while the present study also evaluated benign tumours. In accordance with El Gohary (2008), lymphatic vessels in benign CMTs had the same morphology of those in malignant tumours, regardless of the tumour region.

In contrast to previous human studies, a larger area and perimeter were found in PT tumoural tissue compared to normal mammary gland tissue (Vleugel *et al.*, 2004; Van der Auwera *et al.*, 2005). On the other hand, similar to human

carcinomas, IT regions showed significant lower values for LVD and LVA compared to control tissue (Agarwal *et al.*, 2005; Van der Auwera *et al.*, 2005).

The number of IT and PT lymphatic vessels is the net result of previous phases of tumour lymphangiogenesis and of lymphatic vessel remodelling or regression, which suggests that the measurement of LVD is not necessarily a reflection of the ongoing lymphangiogenesis (Van der Auwera *et al.*, 2006). It has been reported that both lymphangiogenesis and lymphatic hyperplasia play a role in tumour dissemination and are characterized by proliferating lymphatic endothelial cells (Skobe *et al.*, 2001; Stacker *et al.*, 2001). The presence of proliferating lymphatic endothelial cells in human breast cancer, especially in IT regions, is debated (Williams *et al.*, 2003; Agarwal *et al.*, 2005; Van der Auwera *et al.*, 2005; van der Schaft *et al.*, 2007; Kandemir *et al.*, 2012). Absence of proliferating lymphatic endothelial cells or relatively low proliferation rates of these vessels might be due to an inability of the methodology to accurately detect all dividing LECs accurately and therefore may underestimate the real proliferation rate as the sprouting of new vessels begins at different levels among the lymphatics. Moreover, the formation of new lymphatic vessels might not require endothelial mitotic division if they originate from circulating progenitors or non-endothelial cells via transdifferentiation (Mohammed *et al.*, 2009; Ran *et al.*, 2010). In the present study, proliferating LECs were found in IT and PT regions both in benign and malignant CMTs and significant higher proliferation rates were detected compared to control tissue. This suggests the presence of active lymphangiogenesis both in PT and IT regions, similar to findings in human breast cancer (Van der Auwera *et al.*, 2005; Mohammed *et al.*, 2009). However, the higher proliferation status of the LECs in PT versus IT in our study, was not confirmed in human studies (Van der Auwera *et al.*, 2005).

Interestingly, only the LECP in IT regions of benign tumours showed a predictive value, both for DFS and OS. None of the other lymphatic vessel related

parameters showed a significant relation with survival time (OS or DFS). The lack of association between TCP and OS contrasts with previous studies where high Ki67 expression in tumour cells was associated with low DFS and OS and with higher risk of metastasis and death (Vielh *et al.*, 1990; Pena *et al.*, 1998; Sarli *et al.*, 2002; Ferreira *et al.*, 2009; Santos *et al.*, 2013). In human breast cancer research the use of LVD as a prognostic factor for survival also remains controversial. Some studies did not find an association (Kato *et al.*, 2005; van der Schaft *et al.*, 2007; El-Gohary *et al.*, 2008), whereas others were able to correlate LVD with unfavourable survival time (Nakamura *et al.*, 2005; Mohammed *et al.*, 2009; Tsutsui *et al.*, 2010) or to correlate an increase in LVP and LVA with the occurrence of lymphatic metastasis in different human cancers (Dadras *et al.*, 2003; Van der Auwera *et al.*, 2005; Liang *et al.*, 2006). Furthermore, controversy about the role of IT versus PT lymphatic vessels in the pathology of primary human tumours still exists. Several studies have shown that the density of both IT and PT lymphatic vessels is associated with the presence of lymph node metastases (Van der Auwera *et al.*, 2005; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009; Mohammed *et al.*, 2009), while others only described an association between PT LVD and lymph node metastasis (Vleugel *et al.*, 2004; Kandemir *et al.*, 2012). Few studies in other tumours than breast carcinomas have demonstrated that IT and not PT lymphatic vessels are vital for lymphatic metastasis (Beasley *et al.*, 2002; Maula *et al.*, 2003).

The lack of prognostic value in the present study may be caused by a relative high number of missing observations, especially in the IT region and a relative high percentage of animals from which no or insufficient survival data were available. Another possible explanation could be the presence of vasculogenic mimicry, which is the *de novo* generation of microvascular channels by neoplastic cells in highly malignant CMTs (Clemente *et al.*, 2010; Rasotto *et al.*, 2012). It is questioned whether the channels found in CMTs mimic blood vessels, lymphatic vessels or both. The hypothesis that these microvascular channels can promote

the lymphatic spread of tumour cells and consequently negatively influence the survival is plausible. Repeating this analysis using a larger sample size with improved follow-up and with identification of possible vasculogenic mimicry may result in a better estimate of the predictive value of the LV parameters.

The present study has given a detailed description of lymphatic vessel characteristics in CMTs. An improved patient follow-up could achieve more reliable survival data. Most of our findings were in accordance with the results of studies in human breast cancer, which supports the use of dogs with spontaneously occurring CMTs as a model for human breast cancer in comparative oncology trials. As the survival data in this study did not reach significance, further research on a larger number of patients is imperative to evaluate the possible prognostic value of lymphatic vessel parameters.

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CHAPTER 5. Angiogenesis in canine mammary tumours: a morphometric and prognostic study

Based on

Sleeckx N., Van Brantegem L, Van den Eynden G., Fransen E., Casteleyn C., Van Cruchten S., Veldhuis Kroeze E., Van Ginneken C. (2013) Angiogenesis in Canine Mammary Tumours: A Morphometric and Prognostic study. *Journal of Comparative Pathology*, published on line http://dx.doi.org/10.1016/j.jcpa.2013.09.005

5.1. Abstract

Angiogenesis in canine mammary tumours has been described previously; however, only the intratumoural (IT) region has been studied and information on peritumoural (PT) angiogenesis is still lacking. In this study, the blood vessel density (BVD), blood vessel perimeter (BVP) and blood vessel area (BVA) in IT and PT regions of 56 benign canine mammary tumours (CMTs), 55 malignant CMTs and 13 samples of normal mammary gland tissue were analysed. In addition, the blood endothelial cell proliferation (BECP) as an indicator of ongoing angiogenesis was investigated. The prognostic value of each parameter was also examined. Blood vessels and proliferating blood endothelial cells were present in IT and PT regions of both benign and malignant tumours. The vessels in the PT region had a significantly higher area and perimeter compared to those in the IT region. Malignant tumours showed significantly more vessels with a smaller total BVA and a higher BECP compared to benign tumours and control tissue. Interestingly, in the PT regions there was a significantly higher BVD, BVA and BVP compared to the vessels in the control tissue. Only the IT and PT BVD and PT BECP in benign tumours seem to allow a prediction of survival. In conclusion, it can be stated that the morphology of blood vessels in CMTs shows many similarities with those in human breast cancer, which can strengthen the use of dogs with CMTs in comparative oncology trials.

5.2. Introduction

Mammary tumours are the most common neoplasms in women and bitches. During their growth angiogenesis and lymphangiogenesis are induced, since the formation of these new blood and lymphatic vessels originating from the pre-existing vascular network is essential for tumour growth, invasion and metastasis (Folkman, 1986; Fox *et al.*, 1996; Saaristo *et al.*, 2000; Al-Rawi *et al.*, 2005; Sleeman and Thiele, 2009; Holopainen *et al.*, 2011). The characteristics of angiogenesis in human breast tumours and their correlation with metastasis and prognosis have been extensively studied (Weidner *et al.*, 1991; Uzzan *et al.*, 2004).

Likewise, in veterinary medicine, the presence of angiogenesis in different canine tumours including canine mammary tumours (CMTs), has been a subject of interest. However, most of these studies have only focused on the blood vessel density (BVA) in the intratumoural (IT) region (Griffey *et al.*, 1998; Graham and Myers, 1999). Moreover, not all of these studies took into account the area, perimeter and proliferation status of these blood vessels and none of them evaluated peritumoural (PT) regions. As the PT region is also exposed to angiogenic stimulation, investigation of the different blood vessel characteristics in this region can provide interesting additional information. Different blood vessel markers have been used in the past. However, a previous study performed by our group compared different immunohistochemical blood and lymphatic vessel markers in normal and neoplastic canine mammary tissue. CD31 was found to be the most suitable blood vessel marker (Sleeckx *et al.*, 2013), so it was used to investigate the blood vessels in CMTs. The combination of CD31 and the proliferation marker Ki67 was used to assess ongoing angiogenesis. Labelling of lymphatic vessels with prox-1 was performed to discriminate between lymphatic and blood vessels (Van den Eynden *et al.*, 2006).

Since CMTs show similarities to human breast cancer (Khanna and Hunter, 2005; Porrello *et al.*, 2006; Lavalle *et al.*, 2009; Uva *et al.*, 2009; Klopfleisch *et al.*, 2011; Queiroga *et al.*, 2011b; Casteleyn *et al.*, 2013), pet dogs with naturally occurring CMTs represent a valuable animal model in comparative oncology trials. Data from these studies may support the development of anti-angiogenic therapeutics (Rusk *et al.*, 2006; Paoloni *et al.*, 2009). Acquiring more information on blood vessel characteristics in CMTs and comparing these data with findings in human breast cancer is required for the establishment of a comparative oncology model for anti-angiogenic therapy.

The aims of this study were (a) to investigate in detail the blood vessel characteristics in IT and PT regions, both in benign and malignant CMTs, (b) to

examine the proliferation status of tumour-associated blood vessels as an indicator of ongoing angiogenesis and (c) to assess the prognostic value of these blood vessel characteristics.

5.3. Material and Methods

Samples

One hundred and twenty four samples of mammary glands were collected. Samples of healthy canine mammary glands (*n* = 13) were collected during necropsy examination of bitches with normal non-neoplastic mammary glands. These dogs varied in age from 6 to 16 years (mean age 10.75 years) and different breeds were represented. The CMTs (*n* = 111) were surgically removed from female dogs with a mean age of 10 years (age range from 5 to 17 years) and were submitted to the Laboratory of Applied Veterinary Morphology of the University of Antwerp, Belgium. The CMTs were classified according to the World Health Organization's diagnostic criteria (Misdorp *et al.*, 1999). Grading of the malignant tumours was performed according to the Elston and Ellis method adapted to CMTs (Clemente *et al.*, 2010; Pena *et al.*, 2013). Clinical follow-up data (recurrence, metastases and survival) were recorded based on case record, client and veterinarian follow-up for a minimum of 12 months.

Immunohistochemistry

Samples were fixed in 4% neutral buffered formaldehyde, processed routinely and embedded in paraffin wax. Serial sections were stained with haematoxylin and eosin (HE) and for immunohistochemistry (IHC)labelled with CD31 (Dako, Glostrup, Denmark), Ki67 antibody (Dako) and prox-1 antibody (RELIATech, Wolfenbüttel, Germany). A summary of the immunohistochemical protocols can be found in Table 5-1. Three washes with Dako wash buffer were performed between each step of the procedure. Reactions were 'visualised' using 3,3'-diaminobenzidine (DAB, Dako) and counterstaining with haematoxylin was performed. Positive controls included a section of canine haemangioma for CD31

and a section of canine lymph node for prox-1. Blood and lymphatic vessels in normal mammary tissue and in non-tumour areas of the tumour tissue samples served as additional internal controls. Epidermis on the sections was used as internal positive control for Ki67 because of the physiological presence of proliferating keratinocytes in the stratum basale. For negative controls, the primary antibody was replaced with 0.05M TBS containing 0.3% Triton X-100 (Sigma Aldrich, St Louis, Missouri, USA) and 1% bovine serum albumin (Sigma Aldrich).

Assessment of morphological characteristics

All slides (4 x 124, *n* = 496) were evaluated using an Olympus BX61 microscope (Olympus, Aartselaar, Belgium) equipped with an Olympus DP50 digital camera connected to a computer system running the Olympus software program Analysis Pro™. Image analysis was performed without knowledge of clinicopathological details. Microvessel 'hotspots' were identified in HE-stained sections as areas containing numerous microvessels at low magnification (x40 and x100) (Vermeulen *et al.*, 2002). Two non-overlapping hotspots were chosen for IT and PT regions of the tissue. Three non-overlapping hotspots were selected in sections of normal mammary tissue. Digital images of these hotspots were taken at a magnification of x200. In each hotspot, the number of blood vessels per mm² (BVD), the total and average blood vessel perimeter (BVP) and the total and relative area occupied by blood vessels (BVA) were analysed. Evaluation of the microvessels was performed according to the method of Weidner (1991). In addition, the percentage of proliferating blood endothelial cells (BECP) was calculated. The BECP was calculated in each hotspot as the number of blood endothelial cells with KI67 positive stained nuclei per 100 blood endothelial cells.

Table 5-1 *Summary of the immunohistochemical protocols*

	CD31	Ki67	Prox-1
Target	Blood vessel endothelial cells	Proliferating cells	Lymphatic vessel endothelial cells
Antigen Retrieval	HIER microwave, 20 min 90W Dako target retrieval solution Tris-EDTA buffer pH 9.0 (Dako)	HIER microwave, 15 min 90W Dako target retrieval solution citrate buffer pH 6.0 (Dako)	HIER microwave, 15 min 90W Dako target retrieval solution citrate buffer pH 6.0 (Dako)
Primary Antibody	Monoclonal mouse anti-human CD31, clone JC70A (Dako) Dilution 1 in 20 incubation 4°C ON	Monoclonal mouse anti-human Ki67, clone MIB-1 (Dako) Dilution 1 in 250 incubation 60 min 37°C	Polyclonal rabbit anti-human prox-1 (RELIATech) Dilution 1 in 100 incubation 60 min 37°C
Secondary antibody	Polyclonal goat anti-mouse immunoglobulins/ biotinylated (Dako) 1 in 200, 30 min RT	Polyclonal goat anti-mouse immunoglobulins/ biotinylated (Dako) 1 in 200, 60 min RT	Polyclonal goat anti-rabbit immunoglobulins/ biotinylated (Dako) 1 in 200, 30 min RT
Enzyme complex	Streptavidine-horseradish Peroxidase (Dako), 1 in 200, 30 min RT	Streptavidine-horseradish Peroxidase (Dako), 1 in 200, 60 min RT	Streptavidine-horseradish Peroxidase (Dako), 1 in 200, 30 min RT

W, Watts; ON, overnight; RT, room temperature; HIER, heat-induced antigen retrieval

Statistical analysis

Evaluation of blood vessel density, blood vessel area, blood vessel perimeter and blood endothelial cell proliferation

Statistical analyses were performed using the statistical package R (www. R-project.org). Linear mixed models were fitted in the lmer function in the lme4 package in R. All variables were analysed by fitting linear mixed models that included a random intercept term for tumour number, to account for the dependency between observations within the same tumour. As fixed effects, malignancy and the position within the tumour (IT or PT) were entered, as well as the interaction between them. In this model, the interaction between benign and

malignant tumours was first tested. After removing the non-significant interaction term, the model was refitted with only main effects for IT/PT and malignancy and the significance of these two main effects was calculated. Significance of the interaction term and the main effects were tested using a likelihood ratio test (Agresti, 2002). Additional tests to separately compare the controls with the benign and malignant tumours were performed using the same linear mixed model framework.

To compare the BECP values in control tissue versus IT and PT regions, both in malignant and benign tumour samples, pairwise Mann-Withney tests were performed. For IT and PT tissues separately, the difference in the presence of blood vessels and BECP in benign and malignant tumours was tested using the Fisher exact test.

Log-transformed values were used in all analyses, except for the proliferation parameters. $P < 0.05$ was considered statistically significant.

Association of blood vessel density, blood vessel area, blood vessel perimeter and blood endothelial cell proliferation with survival time

Survival analysis were carried out using the software package R, with the coxph function in the survival package. Follow-up data were expressed as disease free survival (DFS) time, defined as the interval between surgery and evidence of local recurrence and/or metastases and as overall survival (OS) time, defined as the interval between surgical treatment and death due to the tumour. In case no recurrence or metastasis (for DFS) or no death due to tumour (for OS) had been observed by the end of the study period, in case the patient was lost to follow-up or a different event making a further follow-up impossible (including death from an unrelated illness (Webster *et al.*, 2011), the last time point without event was recorded and an individual was considered "censored". To find out which parameters had the strongest association with survival time, taking into account the censoring, a Cox proportional hazards regression model was fitted (Cox, 1972). The different blood vessel parameters were entered as covariates. The

predictive accuracy of the model was evaluated using the concordance between the observed order (of the event) and the predicted order. The closer the value is to 1, the better the parameter predicts survival (Gönen and Heller, 2005).

5.4. Results

Different histological types of CMTs were present. Of the 111 tumoural specimens, 56 (50.5%) were benign (simple adenoma (*n* = 14), complex adenoma (*n* = 25), benign mixed tumour *(n* = 17) and 55 (49.5%) were malignant (tubulopapillary carcinoma (*n* = 10), solid carcinoma (*n* = 36), anaplastic carcinoma (*n* = 5), complex carcinoma (*n* = 3), carcinosarcoma (*n* =1)). Grade II tumours were overrepresented (*n* = 29), next to grade I (*n* = 12) and grade III (*n* = 14). About a fifth (23/111) of the dogs was lost to follow-up directly after surgery and therefore, could not be included in survival analysis.

Evaluation of blood vessel density, blood vessel area, blood vessel perimeter and blood endothelial cell proliferation

IT blood vessels were present in 97.2% of benign tumours and in 100% of malignant tumours (*P* = 0.098). PT blood vessels were present in 99% of benign CMTs and in 100% of malignant CMTs (*P* = 0.47). Figure 5-1 shows IT and PT blood vessels and proliferating blood vessel endothelial cells.

Significantly more blood vessels with a higher total perimeter were present in benign and malignant tumours compared to control tissue. The PT blood vessels occupied a larger area and had a larger perimeter than the IT blood vessels (*P* < 0.01). The interaction between malignancy and between IT and PT regions was nowhere significant and this difference was identical in benign and malignant tumours. Malignant tumours showed a significantly larger number of blood vessels with a higher total area compared to benign tumours. Again, as interaction was not significant, this difference was identical in IT and PT regions.

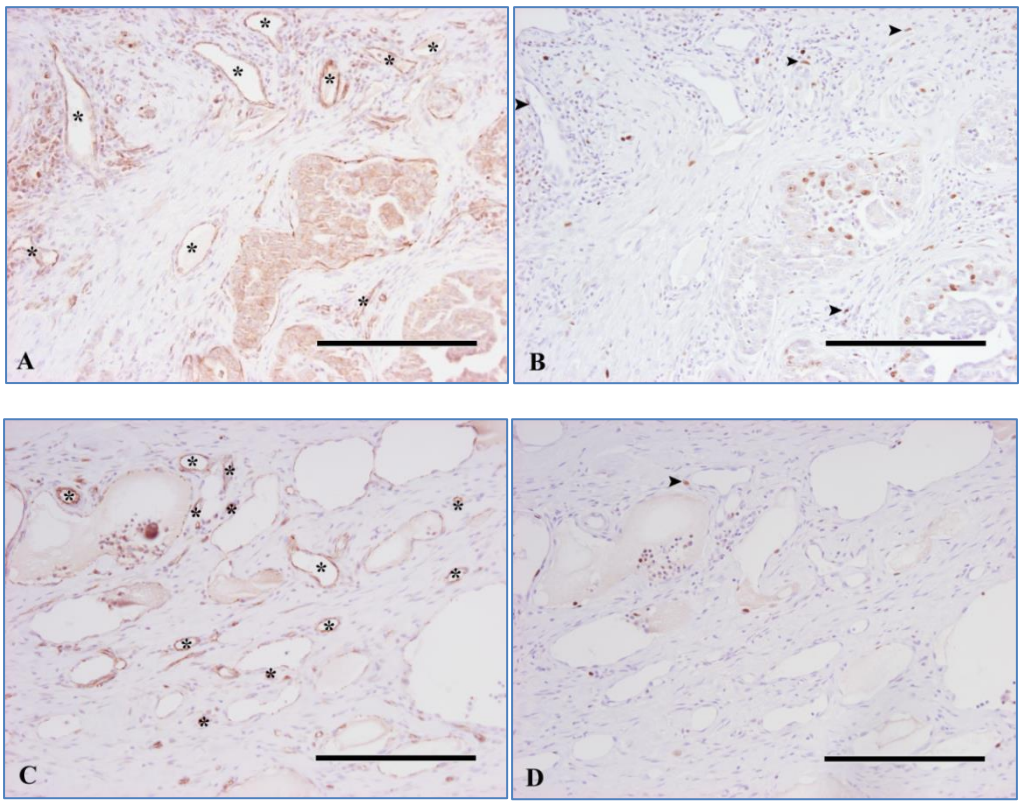

Figure 5-1. *Intratumoural (A and B) and peritumoural (C and D) region of a solid carcinoma with focal tubular differentiation. (A and C) CD31 immunohistochemistry (IHC), (B and D) Ki67 IHC. Blood vessels are indicated with asterisks, proliferating blood endothelial cells with arrowheads. Scale bar, 200 µm*

In line with the parallel study of lymphatic vessels (Sleeckx *et al*, 2013b), additional comparison of the control tissue and the different regions, both in benign and malignant tumours, was made so that comparison with some human breast cancer studies was possible. All different regions (IT and PT, both in benign and malignant CMTs) showed a significantly higher BVD than control tissue. A significantly higher total BVP compared to controls was seen in the IT and PT regions of malignant tumours and in the PT regions of benign tumours. Relative BVA was higher in the PT regions compared to controls and although not significant, the IT blood vessels had a lower relative BVA compared to controls.

In addition, mean BVA was lower in IT regions in benign tumours and total BVA was higher in PT regions in malignant tumours compared to controls.

Proliferating blood endothelial cells were seen in benign and malignant CMTs. IT proliferating blood endothelial cells were present in 32.5% of benign tumours and in 47.2% of malignant tumours (P = 0.059). In PT regions, 22.4% of benign tumours and 48% of malignant tumours (P <0.001) contained Ki67 positive blood endothelial cells. A significant lower BECP was found in controls compared to benign and malignant tumours, although only the comparison with the latter was significant. BECP values differed significantly between the control samples and the different tumour regions, both in benign and malignant tumours. The least significant differences were found between controls and the benign PT region. On correction for multiple testing, the latter pairwise comparison was no longer significant. All other comparisons between control and tumour tissue remained significant on multiple testing correction. A summary of the median value of the blood vessel parameters and their *P*-values can be found in Tables 5-2 and 5-3.

Association of blood vessel density, blood vessel area, blood vessel perimeter and blood endothelial cell proliferation with survival time

Only the BVD and BVP in benign tumours had a predictive value (concordance > 0.8) for DFS. In contrast, the best predictive value for OS was given by BVD in benign tumours, by median area and median perimeter in IT region and by total BVP in PT region of benign tumours (concordance > 0.8). In addition, the BECP in PT benign tumours had a predictive value, both for DFS and OS (concordance > 0.9).

Table 5-2 *Blood vessel related parameters in normal mammary tissue, benign CMTs and malignant CMTs*

Parameter	Control	Benign Tumours	Malignant Tumours	P_1 value	P_2 value
BVD/HPF	9	12	14	**0.019**	**4.7E-04**
BVD/mm²	31.03	41.38	48.27	**0.021**	**5.9E-04**
Total BVA (µm²)	9375.05	12079.19	16426.54	0.73	0.15
Mean BVA (µm²)	1116.50	950.69	1038.67	0.29	0.71
Median BVA (µm²)	318.61	287.70	281.19	0.36	0.55
Relative BVA (%)	3.20	4.12	5.60	0.35	0.06
Total BVP (µm)	1240.45	1811.32	2134.65	**0.035**	**0.002**
Mean BVP (µm)	146.08	137.44	145.01	0.52	0.72
Median BVP (µm)	88.00	92.65	90.77	0.48	0.65
BECP (%)	0	0	0	0.10	**0.007**

Values in table are presented as median, *P*-values in bold are considered significant (< 0.05), BVD, blood vessel density; HPF, high power field 400x magnification; BVA, blood vessel area; BVP, blood vessel perimeter; BECP, blood endothelial cell proliferation; control = normal mammary gland tissue. P_1 value represents the comparison of control tissue versus benign tumours, P_2 value represents the comparison of control tissue versus malignant tumours.

Table 5-3 *Blood vessel related parameters in normal mammary tissue and intra- and peritumoural areas in benign and malignant CMTs*

Parameter	*Control*	*Benign CMTs*		*Malignant CMTs*		P_1 *value*	P_2 *value*	P_3 *value*	P_4 *value*	P_5 *value*	P_6 *value*
		IT	**PT**	**IT**	**PT**						
BVD/HPF	9	13	12	13	16	**0.03**	0.36	**0.01**	**0.03**	**0.002**	**1.10E-04**
BVD/mm²	31.03	44.82	41.38	44.82	55.17	**0.03**	0.25	**0.02**	**0.03**	**0.003**	**1.30E-04**
Total BVA (µm²)	9375.05	8702.45	16061.82	8387.92	26387.14	**0.02**	**6.63E-10**	0.39	0.10	0.98	**1.66E-04**
Mean BVA (µm²)	1116.50	572.77	1388.75	590.40	1716.69	0.20	**2.91E-10**	**0.01**	0.64	0.07	0.26
Median BVA (µm²)	318.61	209.33	355.25	249.94	368.85	0.61	**1.10E-04**	0.05	0.85	0.19	0.88
Relative BVA (%)	3.20	2.97	5.47	2.86	8.99	0.15	**6.76E-12**	0.75	**0.03**	0.93	**1.20E-04**
Total BVP (µm)	1240.45	1650.67	1949.80	1795.14	2576.23	0.06	**1.45E-05**	0.18	**0.006**	**0.04**	**3.00E-05**
Mean BVP (µm)	146.08	117.10	154.89	129.83	165.61	0.60	**7.03E-06**	0.07	0.60	0.24	0.61
Median BVP (µm)	88.00	84.06	95.31	87.39	95.87	0.64	**0.03**	0.17	0.10	0.46	0.90
BECP (%)	0	0	0	0	0	0.07	0.51	**0.003**	**0.03**	**3.50E-05**	**3.00E-05**

Values in table are presented as median, *P*-values in bold are considered significant (< 0.05); IT, intratumoural; PT, peritumoural; BVD, blood vessel density; HPF, high power field 400x magnification; BVA, blood vessel area; BVP, blood vessel perimeter; BECP, blood endothelial cell proliferation; control = normal mammary gland tissue. P_1 value represents the comparison of benign versus malignant tumours (both for IT and PT regions as interaction was not significant, P_2 value represents the comparison of IT regions versus PT region (both for benign and malignant tumours as interaction was not significant), P_3 value represents the comparison of control versus benign intratumoural regions, P_4 value represents the comparison of control versus benign peritumoural regions, P_5 value represents the comparison of control versus malignant intratumoural regions, P_6 value represents the comparison of control versus malignant peritumoural regions.

5.5. Discussion

Although a consensus on the methodology of angiogenesis and lymphangiogenesis quantification in human tumours has been published during the last two decades (Vermeulen *et al.*, 1996; Vermeulen *et al.*, 2002; Van der Auwera *et al.*, 2006), the experimental mode of action in mammary cancer studies differs substantially, which hinders a good comparison of literature data. While the lymphangiogenesis consensus highlights the importance of the evaluation of both IT and PT regions, the angiogenesis consensus does not mention the investigation of the PT area (Vermeulen *et al.*, 1996; Graham and Myers, 1999). Most studies of human and animal tumour-associated angiogenesis do not specify the location of the hotspots. Only a limited number of studies in human medicine (Vartanian and Weidner, 1994; Avdalyan *et al.*, 2012; Hollemann *et al.*, 2012) and none in veterinary medicine describe the evaluation of PT regions. Both regions were evaluated in the present study and interestingly, a difference between PT and IT regions was observed. In both benign and malignant tumours, the vessels in the PT region had a significantly larger area and perimeter compared to those in the IT region. These results are in accordance with findings in some human tumours other than breast cancer (Avdalyan *et al.*, 2012; Hollemann *et al.*, 2012) in which more PT vessels compared to IT vessels showed a distinct lumen and a higher vessel area and perimeter. The present study also demonstrated that IT vessels in malignant tumours were more numerous with a smaller total BVA compared to those in benign tumours, which is in accordance to the veterinary (Griffey *et al.*, 1998; Graham and Myers, 1999; Restucci *et al.*, 2000; Jakab *et al.*, 2008; Queiroga *et al.*, 2011a) and human medicine (Folkman *et al.*, 1989; Uzzan *et al.*, 2004) literature. This decreased vessel area is suggested to be a consequence of the increased angiogenesis, which gives rise to numerous but small and abnormal new blood vessels, often without a distinct lumen. BVD, BVA and BVP of the PT vessels were higher when compared to the blood vessels in the control tissue. This confirms that the vessels in the PT region differ from those in normal

mammary gland tissue. It has been shown that Vascular Endothelial Growth Factor A (VEGFA) and its receptor VEGFR-2 are present within the tumour and in the PT region of CMTs (Restucci *et al.*, 2004; Millanta *et al.*, 2006). In addition, in different human tumours it has been demonstrated that the PT region is exposed to angiogenic influence and can participate in tumour feeding and spread of metastasis (Avdalyan *et al.*, 2012; Mao *et al.*, 2012). These findings were confirmed in the present study, as the PT regions of the malignant tumours contained more vessels, and had a larger BVA and BVP compared to the benign tumours.

Tumour vessel endothelial cells proliferate 30 to 40-fold faster than endothelial cells in normal tissue (Hobson and Denekamp, 1984). In the present study, proliferating blood vessel endothelial cells were found both in IT and PT regions of benign and malignant tumours. In contrast to lymphatic endothelial cells, no difference was found for the proliferation of blood endothelial cells between IT and PT regions. Nevertheless, the number of proliferating blood endothelial cells increased progressively in benign and malignant tumours compared to control tissue. Yet only the comparison of normal tissue versus malignant tumours showed significance. In addition, significance was found for the comparison of the normal mammary tissue with the IT and PT regions of both benign and malignant tumours separately. These findings confirm the presence of active angiogenesis as already shown in IT regions of human breast cancer (Fox *et al.*, 1993; Arnes *et al.*, 2012).

None of the blood vessel parameters were able to predict survival for either benign and malignant tumours. However, for benign tumours there was some association with survival, with the highest concordance observed for the IT BVD, both for DFS and OS. This contrasts with most veterinary studies, which show shorter DFS, OS and/or presence of metastasis in CMT-patients with high BVD (Griffey *et al.*, 1998; Graham and Myers, 1999; Lavalle *et al.*, 2009). Studies of

human breast tumours show contradicting and inconclusive results. Some have found that a high BVD is associated with a higher chance of metastasis and poor survival (Weidner *et al.*, 1991; Fox *et al.*, 1995; Hansen *et al.*, 2000), while other reached the opposite conclusion (Guidi *et al.*, 2000; Gunel *et al.*, 2002). Again, the lack of a universal method compromises the interpretation of the different results (Uzzan *et al.*, 2004). Although the present sample size was larger than that of most previous studies, the lack of prognostic value in the present study may be caused by a relative high percentage of animals from which no or insufficient survival data was available.

Recently, vascular proliferation was suggested as a better prognostic indicator than BVD (Arnes *et al.*, 2012). This may be true for tumours that mainly promote angiogenesis, but some breast tumours and CMTs use additional methods to ensure vascularisation of the tumour and some of them do not induce endothelial cell proliferation (Auguste *et al.*, 2005; Jakab *et al.*, 2008). Alternative methods, which can occur simultaneously, can involve co-option of pre-existing vessels, postnatal vasculogenesis and vasculogenic mimicry (De Spiegelaere *et al.*, 2012). The latter is known to be present in highly malignant breast carcinomas and CMTs and involves the *de novo* formation of microvascular channels by genetically deregulated aggressive tumour cells without endothelial cell participation. As these channels may distribute plasma, blood cells and tumour cells, they can negatively influence the survival time without increasing BVD and BECP (Folberg *et al.*, 2000; Clemente *et al.*, 2010; Rasotto *et al.*, 2012). In the present study, only the PT BECP showed a high concordance for DFS and OS. Repeating this analysis with improved follow up and with identification of non-angiogenic vascularisation with special attention for vasculogenic mimicry, may result in a better estimate of the predictive value of the BV parameters.

In conclusion, we can state that the IT and PT blood vessels in CMTs show many similarities with those in human breast cancer. The present study has reported

characteristics of the PT blood vessels, for the first time in CMTs. Active angiogenesis together with a higher number of blood vessels in malignant versus benign CMTs could be found. In addition, a higher area and perimeter of the PT blood vessels compared to the IT ones was detected. These findings may enhance the application of dogs with spontaneously occurring CMTs in comparative oncology studies.

5.6. References

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CHAPTER 6: Presence of fibrotic foci in canine mammary tumours

Based on

Sleeckx N., Van Brantegem L, Van den Eynden G., Fransen E., Casteleyn C., Van Cruchten S., Veldhuis Kroeze E., Van Ginneken C. Short Paper: presence of fibrotic foci in Canine Mammary Tumours. *Journal of Comparative Pathology,* under revision.

6.1. Summary

Presence of a fibrotic focus (FF) has been described in different human cancers as an indicator of tumour aggressiveness and poor prognosis. To our knowledge, this is the first report of the presence of a FF in canine mammary tumours (CMTs). An association between the presence of FF and high tumour grade and lymphatic vessel density was found in CMTs, but no association with survival time could be demonstrated.

6.2. Introduction

Although canine mammary tumours are the most common neoplasms in intact female dogs, it still remains challenging to put forward a correct prognosis. Fibrotic foci have been described in different human cancers, especially in invasive ductal breast carcinoma, and are used as an indicator of tumour aggressiveness (Van den Eynden *et al.*, 2007). A fibrotic focus (FF) is a scar-like area in the centre of a tumour and can be regarded as a focus of exaggerated reactive stroma formation (Hasebe *et al.*, 1996; Van den Eynden *et al.*, 2007). Consequently, the main components of a FF are fibroblasts and collagen fibres that can be present in varying proportions. A FF can occupy various percentages of the tumour area and is surrounded by a more cellular zone containing invasive tumour cells (Hasebe *et al.*, 1998; Hasebe *et al.*, 2001; Van den Eynden *et al.*, 2007). Within a FF, the fibroblasts and collagen are arranged differently compared with the connective tissue of non-FF breast cancer stroma. Hypoxia, which strongly induces angiogenesis and lymphangiogenesis, is the driving force behind the formation of a FF (Colpaert *et al.*, 2001; Colpaert *et al.*, 2003; Van den Eynden *et al.*, 2007). The importance of a FF in human invasive ductal carcinoma has been analysed extensively. Most studies demonstrate an association of a FF with high histological grade, high frequency of lymph node metastases, high number of distant metastases and high proliferative activity. Moreover a FF significantly contributes to accurately predicting the outcome of patients with invasive ductal breast carcinoma, both for tumour recurrence and survival (Hasebe *et al.*, 1996; Hasebe

et al., 1998; Koyama *et al.*, 1999; Hasebe *et al.*, 2000; Colpaert *et al.*, 2001; Hasebe *et al.*, 2002; Baak *et al.*, 2005; Tamura *et al.*, 2009; Hasebe *et al.*, 2011; Mujtaba *et al.*, 2013). Fibrotic foci can be easily recognised on standard haematoxylin and eosin (HE) stains, which makes them more practical for routine use than immunohistochemistry (IHC), DNA analysis or molecular genetics (Van den Eynden *et al.*, 2007). The aim of this study was to investigate whether fibrotic foci occur in CMTs and if so, are they associated with tumour-characteristics and survival time of CMT-patients.

6.3. Material and Methods

Fifty-five malignant CMTs were surgically removed from female dogs with a mean age of 10 years (age range from 5 to 17 years) and were submitted to the Laboratory of Applied Veterinary Morphology of the University of Antwerp, Belgium. Samples were fixed in 4% neutral buffered formaldehyde, histologically processed, embedded in paraffin and sections were stained with HE. The CMTs were classified according to the World Health Organization's diagnostic criteria (Misdorp *et al.*, 1999) and grading of the malignant tumours was performed according to the Elston and Ellis method adapted to CMTs (Clemente *et al.*, 2010; Pena *et al.*, 2013). Clinical follow-up data (i.e. recurrence, metastases and survival) were obtained from the case records, or from client and veterinarian follow-up for a minimum of 12 months. We applied the consensus methodology as suggested in Van den Eynden *et al*. (2007) and consequently assessed the histological appearance (scar, radiating fibrosclerotic core or irregular moth eaten), location of the FF (centre of tumour or not), the degree of fibrosis (grade 1: large number of fibroblasts with small amount of collagen fibres, grade 3: mainly composed of collagen fibres, mostly hyalinised, grade 2: intermediate between 1 and 3), absolute and relative size, presence of necrosis and/or haemorrhage and presence of tumour cells in the FF. Subsequently, the association of a FF with tumour proliferation, blood- and lymphatic endothelial cell proliferation, tumour size, grade and type, tumour blood vessel (BV) and LV (lymphatic vessel)

parameters (vessel density, vessel surface and perimeter) and survival data (disease free survival (DFS) and overall survival (OS)) was investigated. Analyses were performed using the statistical program R, version 2013.1, with add-on packages lme4 and pbkrtest.

6.4. Results

Different tumour types were present, namely solid carcinoma (*n* = 36), tubulopapillary carcinoma (*n* = 10), anaplastic carcinoma (*n* = 5), complex carcinoma (*n* = 3), carcinosarcoma (*n* =1). Grade II tumours were overrepresented (*n* = 29), next to grade I (*n* = 12) and grade III (*n* = 14). Nineteen tumours (34.5%) contained a FF (Figure 6-1) of which 15 solid tumours, 3 tubulopapillary carcinomas and 1 anaplastic carcinoma. As a consequence, the presence of a FF was not associated with a specific tumour type (*P* = 0.61). In contrast, a significant difference (*P* = 0.0004) in distribution of FF versus non-FF containing CMTs was observed regarding tumour grading. Most of the non-FF CMTs were grade II (61%), 31% were grade I and only 18% were grade III, while 58% of the CMTs with a FF were grade III, 5% were grade I and 37% were grade II. All of the FF were located in the centre of the tumour and most often (68.4%) had a scar-like appearance. In addition, grade II degree of fibrosis was overrepresented (63.2%). The FF had a median absolute size of 5244 µm and a median relative size of 27.16% of the tumour. Haemorrhage and necrosis were present in about half of the fibrotic foci (58%). Most FF contained tumour cells at their borders with non-FF tumour tissue. No association between the presence of a FF and tumour proliferation (*P* = 0.30, 2-sided t-test) or blood and lymphatic vessel proliferation (*P* = 0.69 and 0.15 respectively, Mann-Withney U test) was found. Furthermore, no significant difference in tumour size was observed between FF and non-FF tumours (*P* = 0.14, 2-sided t-test). When analysing the association of the presence of a FF with the tumour BV and LV parameters, the only important difference that was observed between FF and non-FF CMTs was a lower number and total perimeter of LV in FF compared to non-FF CMTs (*P* = 0.04 and 0.02 respectively,

linear mixed model adjusting for differences between IT and PT vessels). The difference in total number and perimeter was observed regardless of the IT or PT location of the vessels (*P* =0.4 and 0.12, interaction test in linear mixed model. Moreover, the DFS, nor the OS was significantly different between FF and non-FF tumours.

6.5. Discussion

To the best of our knowledge, this is the first report of the presence of fibrotic foci in CMTs. About a third of the malignant tumours investigated in the present study, contained a FF and most of them were present in solid carcinomas. This can be explained by the expansive growth pattern of these tumours, which almost certainly will induce hypoxia in central tumour areas. Interestingly, more than half of the FF-containing tumours was grade III, compared to grade II in non-FF CMTs. While in most human invasive breast cancer studies the presence of a FF is associated with more aggressive tumour characteristics and with a shorter DFS and OS (Hasebe *et al.*, 2000), the present study only showed a significant association with high tumour grade but failed to show an association with survival. In addition, differences were found in lymphatic vessel density and total area. In contrast to the findings in human studies, the CMTs with FF showed lower values compared to non-FF CMTs regarding these parameters . Additional research with a larger sample size with inclusion of only one type of CMT, e.g. solid carcinomas, is necessary to elaborate the knowledge on characteristics and prognostic significance of fibrotic foci in CMTs as they might be an easily accessible additional prognostic marker.

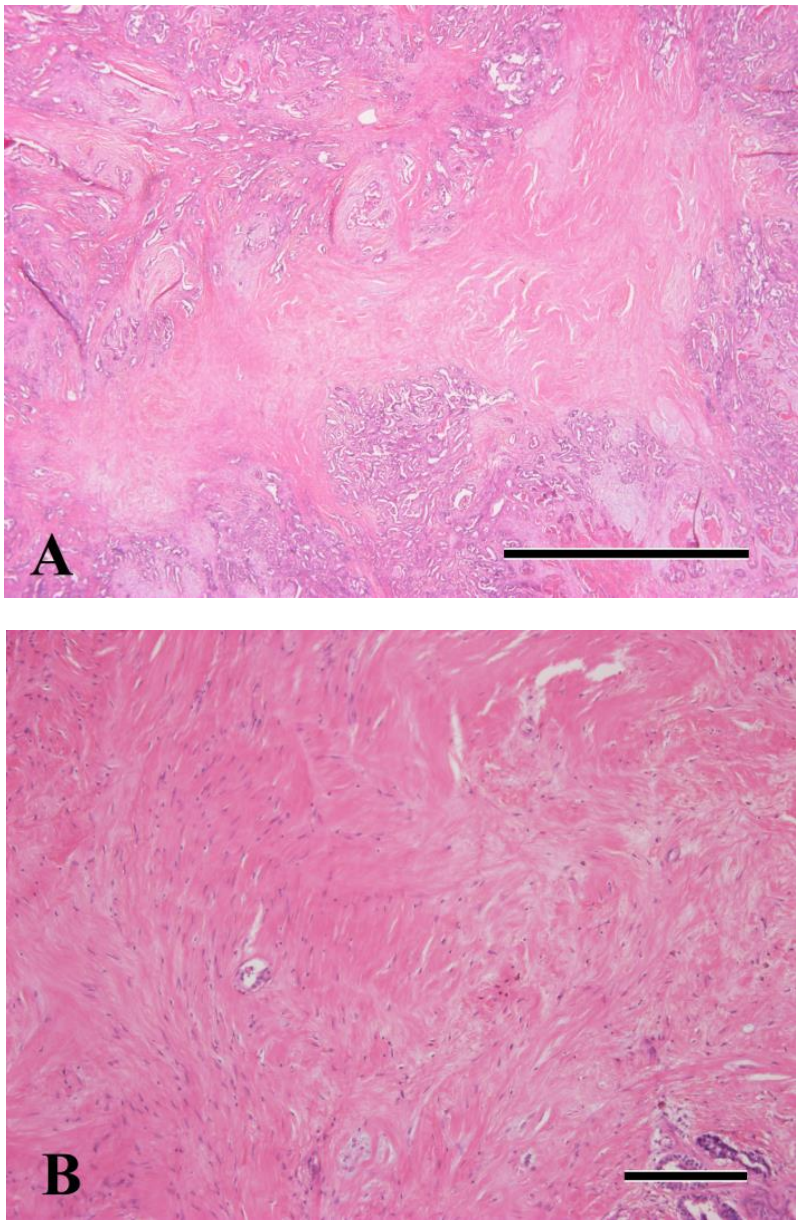

Figure 6-1 *A. Low magnification of a fibrotic focus in a canine mammary carcinoma. Scale bar, 2000 µm. B. Higher magnification showing that the fibrotic focus consists of fibroblast and collagen fibres. Scale bar, 200 µm*

6.6. References

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CHAPTER 7: General Discussion

Mammary tumours still have a high prevalence in bitches and in women (MacEwen and Withrow, 1996). Although an early recognition may lead to a higher survival rate, prognosis and therapy protocols still need improvement. While extensive research showed a relationship between (lymph)angiogenesis, malignancy and prognosis in human breast cancer (Weidner *et al.*, 1991; Uzzan *et al.*, 2004; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009), the induction and role of newly formed blood and lymphatic vessels in CMTs needs further elucidation. In addition, if similarities between canine and human mammary cancer are present, this supports the consideration of dogs with CMTs as an animal model for human breast cancer.

The general aim of this PhD study was therefore twofold. First, we wanted to investigate the characteristics of blood and lymph vessels in CMTs and evaluate whether this would facilitate prognosis. Second, we wanted to examine if the situation in dogs is similar to that in humans in view of a new comparative oncology animal model.

A first step for blood and lymphatic vessel analysis, is the correct visualisation of these vessels (**Chapter 3)**. Immunohistochemistry was preferred because it is a reasonable cheap, readily available and easily assessable method. The ideal immunohistochemical markers should have a high sensitivity and specificity, show low background staining and detect all vessel segments during disease progress (Van der Auwera *et al.*, 2006; Baluk and McDonald, 2008). At the time of the study, no antibodies directed at canine antigens were commercially available. Therefore, we chose to use antibodies directed towards human blood endothelial cells (CD31, CD34 and vWf) and towards human lymphatic endothelial cells (lyve-1, prox-1, podoplanin and D2-40) that have been successfully applied in human breast cancer studies. A number of these blood vessel markers has already been described in different canine tumours, including CMTs (Restucci *et al.*, 2000; Preziosi *et al.*, 2004; Clemente *et al.*, 2010). However, the methodology of these

studies differed significantly, which hindered a complete evaluation. On the other hand, only a few veterinary studies have used the lymphatic markers lyve-1 and prox-1 and none evaluated D2-40, which was proven to be the best markers for lymphatics in human breast cancer (Galeotti *et al.*, 2004; Staszyk *et al.*, 2005; Van der Auwera *et al.*, 2005; Sugiyama *et al.*, 2007; Martin *et al.*, 2010). In the present study, the different blood and lymphatic markers were applied to serial sections of normal and tumoural canine mammary tissue, which allowed a detailed comparison. Antibody titration and epitope retrieval experiments were performed. Endothelial cell labelling could not be achieved for podoplanin, D2-40 and CD34 in the formalin-fixed and paraffin wax-embedded tissues that were available. However, this does not exclude a possible application of these antibodies on cryostat sections. A comparison of CD31 vs. vWf and of prox-1 vs. lyve-1 according to 6 parameters, showed CD31 and prox-1 to be the best blood and lymphatic vessel marker, respectively. Prox-1 reproducibly labelled lymphatic vessels with limited background staining. A disadvantage of the staining might be the fact that the immunoreactivity is nuclear, which can interfere with an intense haematoxylin counterstaining or with a double immunostaining. A moderate haematoxylin counterstain may counter the first problem. CD31 scored best for all parameters, except for specificity. Yet, ideally lymphatic and blood vessel markers need to be combined to discriminate both vessel types, so this lower specificity of CD31 will not cause any problems.

Subsequently, these antibodies were used in a morphometric and prognostic study of lymphangiogenesis (**Chapter 4**) and angiogenesis (**Chapter 5**) in benign and malignant CMTs. Both in intratumoural and peritumoural regions, blood and lymphatic vessel characteristic as well as tumour and endothelial cell proliferation were investigated. Ideally the proliferation marker Ki67 was combined with the prox-1 antibody in a double immunostaining. Yet, as both show nuclear immunoreactivity, interpretation was problematic. Therefore, serial sections were made. In addition, the prognostic value of the analysed parameters was

evaluated. Finally, the blood and lymphatic vessel characteristics in CMTs were compared with the vasculature in human breast cancer.

Although lymphatic vessels are considered as the main route for CMT metastasis, the number, morphology, distribution and prognostic value of lymphatic vessels in CMTs have not been investigated before. The present study analysed this for the first time (**Chapter 4**). Lymphatic vessels were found in PT and IT regions, yet IT lymphatic vessels could only be labelled in about half of the tumours. In human studies, IT lymphatic vessels were present in 0% to more than 80% of the analysed breast tumour samples (Williams *et al.*, 2003; Vleugel *et al.*, 2004; Agarwal *et al.*, 2005; Van der Auwera *et al.*, 2005; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009; Mohammed *et al.*, 2009; Kandemir *et al.*, 2012). This made some authors doubt on the presence of IT lymphatic vessels (Williams *et al.*, 2003; Vleugel *et al.*, 2004; Agarwal *et al.*, 2005). Possible explanations of these varying results may be the heterogeneous nature of the tumour types examined, the variable patient population and the variety in methodology used (El-Gohary *et al.*, 2008; Kandemir *et al.*, 2012). Most studies that failed to detect IT lymphatic vessels used the lyve-1 antibody instead of the prox-1 or podoplanin/D2-40 antibodies. A study that compared lyve-1, prox-1, D2-40 and another anti-podoplanin antibody in human breast cancer samples (Van der Auwera *et al.*, 2005) found large differences in labelling of lymphatic vessels and confirmed that lyve-1 was not able to visualise IT lymphatics in contrast to D2-40, podoplanin and prox-1. D2-40 antibody showed the strongest immunoreactivity with lymphatic vessel endothelium. Unfortunately, D2-40 did not label the lymphatic vessels in CMTs in the present study. In addition, Van der Auwera *et al.* (2005) showed that although a significant correlation of the number of prox-1 stained vessels with the number of D2-40 stained vessels was present, prox-1 stained less lymphatic vessel compared to D2-40. Therefore, we can presume that also in our study we might have underestimated the real number of lymphatic vessels. In addition, we should bear in mind that a difference in immunoreactivity between benign and malignant

tumours might be present. It has been shown that lyve-1 expression can be downregulated in response to inflammation (Johnson *et al.*, 2007), which can be induced in tumours, with the highest response in high grade malignant tumours (Queiroga *et al.*, 2010; Krol *et al.*, 2011; Raposo *et al.*, 2012; Clemente *et al.*, 2013). This might partially explain the lack of prognostic value of the lymphatic vessel related parameters in our study. It is not clear if other factors e.g. growth factors that are upregulated or even downregulated with increasing malignancy (Restucci *et al.*, 2002; Millanta *et al.*, 2010), can influence the immunoreactivity of lymphatic vessel markers. If such differences in immunoreactivity exist, this should be taken into account when concluding on the LVD, as the real LVD could then be different from the measurable LVD. Semiquantitative studies, such as western bot and PCR, may have their value here, but will make the comparison of IT and PT regions difficult. Other possible reasons for the lack of prognostic value, which may also apply to the angiogenesis study, may be the relative high percentage of animals from which no or insufficient survival data were available and the possible presence of vasculogenic mimicry. The latter is the *de novo* generation of microvascular channels by neoplastic cells in some highly malignant CMTs (Clemente *et al.*, 2010; Rasotto *et al.*, 2012). It is questioned whether the channels found in the CMTs mimic blood vessels, lymphatic vessels or both. The hypothesis that these microvascular channels can promote the lymphatic spread of tumour cells and consequently negatively influence the survival is plausible. Repeating the analyses using a larger sample size with improved follow-up and with identification of possible vasculogenic mimicry may result in a better estimation of the predictive value of the LV parameters.

The present study did not show any significant difference between benign and malignant tumours for the LVD, yet a higher LVA and LVP was found in malignant CMTs compared to control tissue. Interestingly, similarly with human breast tumours (Van der Auwera *et al.*, 2005; El-Gohary *et al.*, 2008; El-Gendi and Abdel-Hadi, 2009), the vessels in the PT regions were more numerous and larger

compared to the IT vessels. When performing the analyses for the PT and IT regions separately, larger LVA and LVP in PT regions and lower LVD and LVA in IT regions compared to control tissue were found.

As the number of lymphatic vessels does not necessarily represents the ongoing lymphangiogenesis, Ki67 IHC was performed. Proliferating LECs in PT and IT regions of benign and malignant CMTs were seen and significantly higher proliferation rates were detected in tumours compared to control tissue. Controversy exists on the presence of proliferating lymphatic endothelial cells in human breast cancer, especially in IT regions (Williams *et al.*, 2003; Agarwal *et al.*, 2005; Van der Auwera *et al.*, 2005). As discussed above, methodology may be responsible for the varying results found. In addition, as the sprouting of new vessels begins at different levels along the lymphatic vessels, not all dividing LECs will be present in a certain tumour slide. Moreover, the formation of new lymphatic vessels might not require endothelial mitotic division if they originate from e.g. circulating progenitors or vessel co-option (Mohammed *et al.*, 2009; Ran *et al.*, 2010).

Angiogenesis has already been investigated in different canine tumours, including CMTs (Griffey *et al.*, 1998; Restucci *et al.*, 2000; Queiroga *et al.*, 2011a). Although both the IT and PT region are under angiogenic stimulation (Restucci *et al.*, 2004; Mao *et al.*, 2012), most of these studies, even human ones, neglected the PT region. Clemente (2013) described the evaluation of regions at the periphery of the tumour, but did not systematically evaluate and compare PT and IT regions. Therefore, we analysed both IT and PT regions for a variety of blood vessel characteristics (**Chapter 5**). In almost all tumours, IT and PT blood vessels were labelled with CD31. Comparable to the veterinary (Griffey *et al.*, 1998; Graham and Myers, 1999; Restucci *et al.*, 2000; Jakab *et al.*, 2008; Queiroga *et al.*, 2011a) and human medicine (Folkman *et al.*, 1989; Uzzan *et al.*, 2004) literature, IT vessels in malignant tumours were more numerous with a smaller BVA compared

to those in benign tumours. Peritumoural vessels showed higher BVD, BVA and BVP compared to the blood vessels in the control tissue, with a higher BVD, BVA and BVP in malignant CMTs compared to benign ones. These findings confirm that the PT region differs from normal mammary tissue and that the PT region is exposed to angiogenic influences and can probably participate in tumour feeding and metastasis. In accordance with human studies that did investigate the PT region (Avdalyan *et al.*, 2012; Hollemann *et al.*, 2012), PT vessels had a significantly larger BVA and BVP compared to the IT ones, both in benign and malignant CMTs. Moreover, a recent study in CMTs showed a decreasing number of vessels (blood and lymphatic vessels) towards the core of the tumour (Clemente *et al.*, 2013).

Ongoing angiogenesis could be found both in IT and PT regions of benign and malignant tumours. However, in contrast to lymphatic endothelial cells, no difference was found for the proliferation of blood endothelial cells between IT and PT regions. Nevertheless, the number of proliferating blood endothelial cells increased progressively in benign and malignant tumours compared to control tissue. Like in lymphangiogenesis, additional tumour vascularisation methods can be present without endothelial cell proliferation (Auguste *et al.*, 2005; Jakab *et al.*, 2008). Alternative methods, which can occur simultaneously, can be co-option of pre-existing vessels, postnatal vasculogenesis and vasculogenic mimicry (Dome *et al.*, 2007).

As in the lymphatic vessel study, none of the blood vessel parameters was able to predict survival for both benign and malignant tumours. This contrasts with most veterinary studies, which show shorter DFS, OS and/or presence of metastasis in CMT-patients with high BVD (Griffey *et al.*, 1998; Graham and Myers, 1999; Lavalle *et al.*, 2009). Similarly to lymphangiogenesis studies, in human breast cancer (Weidner *et al.*, 1991; Guidi *et al.*, 2000; Hansen *et al.*, 2000) contradicting reports are present. Again, the lack of a universal method compromises the

interpretation and comparison of the different studies. As mentioned before, the large number of censored patients in the survival analysis in the present study can partly explain the low prognostic significance. In addition, alternative vascularisation methods might be important in certain tumour types.

When investigating the CMT tissue for angiogenesis and lymphangiogensis, we observed in some tumours scar-like areas similar to the fibrotic foci in human breast cancer (Van den Eynden *et al.*, 2007; Van den Eynden *et al.*, 2008). To our knowledge, this has not been described in CMTs. About a third of the malignant CMTs contained a FF. Most of these tumours were solid carcinomas, which often contain hypoxic regions caused by their expanding growth pattern. It has been described before that hypoxia is the driving force for the formation of a FF (Colpaert *et al.*, 2001a). The present study showed a significant association between the presence of a FF and high tumour grade. However, in contrast to human breast cancer studies (Hasebe *et al.*, 2000; Colpaert *et al.*, 2001b), no association with survival was present. Again, the low sample size and limited survival data might be responsible for these negative results. Although this study has its value in screening and reporting for the first time the presence of FF in CMTs, additional research with a larger sample size with inclusion of only one type of CMT, e.g. solid carcinomas, is necessary to elaborate the knowledge on characteristics and prognostic significance of fibrotic foci in CMTs. Because they can be recognised on the routine HE stains, a FF might be a practical, low cost and easily recognisable prognostic marker.

The many similarities of the studied blood vessel and lymphatic vessel characteristics will stimulate the use of pet dogs with spontaneously occurring CMTs as an animal model in comparative oncology trials for anti-(lymph)angiogenic therapeutics. The present results reinforce the known value of canine mammary tumours as a model for human breast cancer especially in drug development studies (Lindblad-Toh *et al.*, 2005; Uva *et al.*, 2009; Mohammed *et*

al., 2011; Queiroga *et al.*, 2011b; Pinho *et al.*, 2012). The purpose of this model is not to replace the conventional and necessary studies with purpose-bred research mice and Beagles, but to be an intermediary between conventional preclinical models and the human clinical trial and between the different clinical trial phases (Hansen and Khanna, 2004; Khanna and Gordon, 2009). Like the other animal models, the comparative oncology animal model has its advantages and disadvantages. Yet, the strengths and weaknesses of each model can be found at different levels. Limitations that can be seen in rodents or research Beagle dogs can be absent in comparative oncology models and vice versa. Also, what can be a disadvantage in a certain stage, may be an advantage later. For example, the heterogeneity of the pet dog study population can be seen as an disadvantage in early preclinical phase, where there will be a need for a homogenous population to study e.g. the mode of action. However, it may account for the heterogeneity of the human target population in e.g. toxicity studies. It is clear that the strength of using different models lies in the fact that they are complementary, which allows them each to give additional information that cannot be obtained otherwise.

Conclusions

After the establishment of IHC protocols for CD31 and prox-1, respectively blood and lymphatic vessel markers, detailed investigation of both vessel types in CMTs was performed. IT and PT blood and lymphatic vessels were found in CMTs and higher PT and IT BVD and PT LVD could be found in CMTs compared to normal mammary tissue. The PT contained more lymphatic vessels compared to the IT region, while no significant difference was seen for the number of blood vessels. Proliferating blood and lymphatic endothelial cells were found in IT and PT regions, suggesting the presence of ongoing (lymph)angiogenesis. In some tumours, fibrotic foci were present. Unfortunately, prognostic significance was not reached for most of the characteristics, although for some factors trends could be seen. Larger scale studies are mandatory to confirm these trends.

Analysis of blood vessels, lymphatic vessels and FF can be performed on formalin fixed, paraffin embedded tissue and therefore are easy and low-cost parameters. Both the blood and lymphatic vessel characteristics showed many similarities between canine and human mammary tumours, which enhances the use of pet dogs with CMTs as animal models in comparative oncology studies.

Future perspectives

Albeit the sample size of the present study was more than sufficient compared to most of the previously performed IHC studies in veterinary and human literature, a larger sample size in combination with separate analyses per tumour types might be recommended for future research. The present study suffered from a high number of censored data in the survival analysis, which negatively affected the results. Preferentially, such study should be performed under university clinic circumstances with direct and intimate contact of the researcher, veterinary surgeon/oncologist/radiologist/pathologist and patient for a number of reasons. First, supervison of a board certified veterinary surgeon, oncologist, radiologist and pathologist will ensure state-of-the-art diagnostic and therapeutic work-up and follow-up of the patient under reproducible circumstances. Second, direct contact will restrict incomplete patient and tumour files. Third, being present at the clinic during tumour surgery, will make it possible to collect fresh frozen samples of the tumours. Fourth, each patient needs to be seen every 3 months for a detailed examination, including thorax radiographs and abdominal ultrasound, this for at least 2 years after initial treatment. Fifth, close contact with the pathologist enables a complete necropsy when dogs die of tumour or non-tumour related cause. This approach will decrease the number of censored and lost to follow-up patients. Finally, identification and role of possible alternative vascularisation methods, including vasculogenic mimicry need to be investigated.

For FF as well as for angiogenesis and lymphangiogensis prognostic studies it might be interesting to account for the histologic grade and tumour type and to

evaluate if difference between the grades and types exist for the different characteristics that have been investigated in this doctoral thesis. Our sample size was too limited to make this subdivision.

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Summary

As mammary tumours in bitches and women remain an important disease entity, there is a constant need for additional and novel therapeutics and reliable prognostic markers. While extensively researched in human breast cancer, the presence and role of tumour induced angiogenesis and lymphangiogenesis in CMTs needs further investigation. This in view of ***a)*** possible new prognostic factors for dogs with CMTs and ***b)*** the establishment of a new comparative oncology model for human breast cancer research.

Chapter 1 starts with a review of the current knowledge on canine mammary tumours, which underlines the importance of CMTs and the lack of therapeutic protocols next to surgery and, very importantly, reliable additional prognostic factors. In view of the aims of this doctoral research, a summary on tumour vasculature with special attention to angiogenesis and lymphangiogenesis in human and canine mammary tumours was given. Subsequently, the presence of a fibrotic focus in human breast cancer is discussed. In addition, the concept of comparative oncology is covered and the similarities between canine and human tumours, including mammary tumours are highlighted. **Chapter 2** gives an overview of the specific aims of the present thesis.

While some studies already examined angiogenesis in different canine tumours, inconsistencies in study design impeded a complete evaluation. To date, lymphangiogenesis in CMTs has not yet been studied. Thus, immunohistochemical protocols for both blood and lymphatic vessels were developed (**Chapter 3**). Comparison of the most commonly used antibodies against blood (vWf, CD31 and CD34) and lymphatic vessels (lyve-1, prox-1, podoplanin and D2-40) in human and veterinary studies was performed according to a number of parameters. CD31 and prox-1 were determined to be the most suitable markers for blood and lymphatic vessels, respectively.

Chapter 4 aimed to investigate in detail lymphatic vessels in intratumoural and peritumoural CMT regions and to evaluate the prognostic value of these

characteristics. In CMTs, prox-1 labelled lymphatic vessels in both regions. The peritumoural lymphatic vessels were more numerous and larger compared to the intratumoural vessels. Compared to normal mammary tissue, larger area and perimeter in peritumoural regions and lower density and area in intratumoural regions of both benign and malignant tumours was seen. Proliferating lymphatic endothelial cells were found in both regions, demonstrating the presence of ongoing lymphangiogenesis. Higher proliferation rates were seen with increasing malignancy. Most of these findings are in accordance with the findings in human breast cancer. None of the lymphatic vessel characteristics showed associated with survival time, probably due to the large number of censored survival data.

Parallel with the lymphangiogenesis study, the angiogenesis in CMTs was examined (**Chapter 5**). CD31 labelled peritumoural and intratumoural blood vessels in almost all tumours. Blood vessel density was higher in malignant CMTs compared to benign CMTs and in benign tumours compared to control tissue. A higher density, area and perimeter of the peritumoural area compared to the normal mammary tissue were found, confirming the difference between the peritumoural region and normal mammary tissue. This should encourage the evaluation of the peritumoural region in future research. In accordance with the literature, the intratumoural region showed higher blood vessel density with a decreased area. When comparing peritumoural and intratumoural regions, a significantly larger area and perimeter could be found in peritumoural regions, both in benign and malignant CMTs. Comparable to lymphangiogenesis, ongoing angiogenesis was seen in intratumoural and peritumoural regions. As for lymphangiogenesis, higher proliferation rates were found with increasing malignancy. Most of the above discussed results display resemblance with the situation in human breast cancer. Similar to the lymphangiogenesis study, none of the blood vessel characteristics showed prognostic value for all tumour types. As both studies were performed on the same patient population, the limited survival data might have caused this.

During the angiogenesis and lymphangiogenesis analyses, scar-like areas were found in about one third of the malignant tumours. These areas resemble the previously described fibrotic focus in human breast cancer. Most of the tumours that contained a fibrotic focus were grade III tumours. No association with survival could be found (**Chapter 6**).

The many similarities of the blood and lymphatic vessels in canine mammary tumours with the vasculature in human breast cancer, represent an additional justification for the use of dogs with spontaneously occurring mammary tumours as an animal model for human breast cancer.

In conclusion, angiogenesis and lymphangiogenesis were present in both peritumoural and intratumoural regions of benign and malignant CMTs. Higher blood vessel density was described in peritumoural and intratumoural regions of CMTs compared to normal mammary tissue and higher lymphatic vessel density was described in peritumoural regions compared to control tissue. In addition, the peritumoural area contained more lymphatic vessels compared to the intratumoural region. Almost none of the parameters showed association with survival time, probably due to the large number of censored survival data. In about one third of the malignant tumours, fibrotic foci were present. Although an association with high tumour grade and with lymphatic vessel density was found, no association with survival time could be demonstrated. Further research is necessary to evaluate a possible prognostic value of the blood and lymphatic vessel parameters and of FF. However, if the predictive value can be demonstrated, these are reasonable cheap and easily assessable histological prognostic parameters. The morphological data of blood vessels, lymphatic vessels and fibrotic foci are in accordance with the situation in human breast cancer, which enhances the use of dogs with spontaneously occurring CMTs as an animal model in comparative oncology trials.

Samenvatting

Aangezien melkkliertumoren bij de teef en borstkanker bij de vrouw nog steeds veelvoorkomend zijn en de huidige behandelingsmethoden niet altijd een antwoord bieden, is er een constante vraag naar nieuwe en aanvullende behandelingsmethoden en betrouwbare prognostische merkers. Angiogenese en lymfangiogenese bij humane borsttumoren werden al uitvoerig bestudeerd. Er is echter nood aan studies die het voorkomen en de rol van tumor-geïnduceerde angiogenese en lymfangiogenese in melkkliertumoren bij de hond onderzoeken Dit met het oog op a) het vinden van nieuwe prognostische factoren voor honden met melkkliertumoren en b) het bepalen van een nieuw 'comparative oncology'-diermodel voor humaan borstkankeronderzoek.

Hoofdstuk 1 start met een overzicht van de huidige kennis van melkkliertumoren bij de hond. Deze literatuurstudie benadrukt het huidig belang van melkkliertumoren bij de hond en wijst op het gebrek aan behandelingsmogelijkheden naast de chirurgie alsook het gemis aan betrouwbare prognostische factoren. In het kader van de doelstellingen van deze doctoraatsthesis, werd er vervolgens een overzicht gegeven van de tumor vasculatuur met speciale aandacht voor de angiogenese en lymfangiogenese in melkkliertumoren. Aansluitend werd het voorkomen van een fibrotische focus in humaan borstkanker besproken. Tot slotte werd de 'comparative oncology' uitgediept waarbij de overeenkomsten tussen tumoren, waaronder melkkliertumoren, bij de hond en de mens werden benadrukt. De doelstellingen van deze thesis werden weergegeven in **hoofdstuk 2**.

Hoewel angiogenese al in verschillende tumoren bij de hond, waaronder melkkliertumoren, werd bestudeerd, bestaan er nog steeds onduidelijkheden, mede door de sterke verschillen in proefopzet. Lymfangiogense werd tot op heden niet bestudeerd in de veterinaire oncologie. Als eerste stap van dit doctoraatsonderzoek werden er immunohistochemische protocollen opgesteld voor het aankleuren van bloed en lymfevaten in caniene melkkliertumoren

(**Hoofdstuk 3**). De meest gebruikte antilichamen voor het immunohistochemisch aankleuren van bloedvaten (vWF, CD31 en CD34) en lymfevaten (lyve-1, prox-1, podoplanine en D2-40) in humane en veterinaire studies werden met elkaar vergeleken volgens een aantal parameters. CD31 en prox-1 bleken de meest geschikte merkers te zijn voor bloed- en lymfevaten, respectievelijk.

Vervolgens werden de lymfevaten in intratumorale en peritumorale regio's van melkkliertumoren bij de hond in detail bestudeerd (**Hoofdstuk 4**). Tevens werd de prognostische waarde van deze karakteristieken nagegaan. Prox-1 kleurde lymfevaten aan in beide regio's, maar de peritumorale lymfevaten waren talrijker en groter in vergelijking met de intratumorale lymfevaten. In vergelijking met de normale melkklierweefsel vertoonden zowel benigne als maligne tumoren grotere lymfevatoppervlakte en -omtrek in de peritumorale regio's en lagere densiteit en oppervlakte in de intratumorale regio's. De aanwezigheid van prolifererende lymfevatendotheelcellen in beide regio's toont aan dat er effectief lymfangiogenese wordt geïnduceerd. Hogere proliferatieratio's werden gezien met toenemende maligniteit. De meeste van deze bevindingen zijn vergelijkbaar met de situatie in humane borsttumoren. Geen enkel lymfevatkenmerk vertoonde een associatie met overlevingstijd. Het grote aantal gecensureerde overlevingsdata kan de oorzaak hiervan zijn.

Parallel met de lymfangiogenesestudie werd de angiogenese in caniene melkkliertumoren onderzocht (**Hoofdstuk 5**). CD31 kleurde intra- en peritumorale bloedvaten aan in zo goed als alle tumoren. Het aantal bloedvaten was hoger in tumoraal weefsel vergeleken met het normale melkklierweefsel en was het hoogste in maligne tumoren. De peritumorale regio vertoonde een hogere bloedvatdensiteit, -oppervlakte, en -omtrek vergeleken met het normale melkklierweefsel. Dit bevestigt dat het peritumorale gebied duidelijk verschillend is van het normale melkklierweefsel en dus eveneens onder pro-angiogenetische invloed staat. Deze bevindingen zouden het evalueren van de peritumorale regio

in toekomstige studies moeten stimuleren. Overeenkomstig met de literatuur vertoont de intratumorale regio meer bloedvaten met een kleinere oppervlakte vergeleken met normaal melkklierweefsel. Indien we vergelijken met de peritumorale regio, dan vertonen deze peritumorale bloedvaten echter een grotere oppervlakte en omtrek, zowel in benigne als in maligne tumoren. Analoog met de lymfangiogenese, werden er prolifererende bloedvatendotheelcellen aangetoond in intra- en peritumorale regio's. Ook hier werden hogere proliferatieratio's gevonden met stijgende maligniteit. Het merendeel van deze resultaten vertonen gelijkenissen met de angiogenese in humaan borstkanker. Net zoals bij de lymfangiogenesestudie vertoonden geen enkele parameter prognostische waarde. Beide studies werden uitgevoerd op dezelfde studiepopulatie, dus ook hier kunnen de beperkte opvolging data de oorzaak zijn.

Tijdens de angiogenese- en lymfangiogenesestudies werden er littekenachtige zones opgemerkt in ongeveer één derde van de maligne tumoren. Deze zones lijken erg op de eerder beschreven fibrotische foci in humane borsttumoren. De meeste tumoren die een fibrotische focus bevatten, zijn graad III tumoren. Ook hier kon er geen associatie met overleving konden aangetoond.

De vele gelijkenissen van de bloed- en lymfevaten in caniene melkkliertumoren met deze in humane borsttumoren, zijn een extra verantwoording voor het gebruik van de huisdierhond met spontane melkkliertumoren als diermodel voor humaan borstkankeronderzoek.

In conclusie kunnen we zeggen dat zowel angiogenese als lymfangiogenese aanwezig is in peri- en intratumorale zones van benigne en maligne caniene melkkliertumoren. Een hogere peri- en intratumorale bloedvatdensiteit en hogere peritumorale lymfevatdensiteit werd aangetoond in vergelijking met normaal melkklierweefsel. De peritumorale zone vertoonde ook meer lymfevaten ten opzichte van de intratumorale zone. Bijna geen enkele parameter vertoonde een associatie met de overlevingstijd, waarschijnlijk door het grote aantal

gecensureerde overlevingsdata. In ongeveer een derde van de maligne tumoren waren fibrotische foci aanwezig. Hoewel er een associatie met tumor graad aanwezig was, kon er geen associatie met overlevingstijd worden aangetoond. Bijkomend onderzoek is nodig om het mogelijk prognostisch belang van de FF en bloed- en lymfevatmerkers aan te tonen. Echter, als een voorspellende waarde van deze parameters kan worden aangetoond, dan zijn dit relatief goedkope en makkelijk beoordeelbare histologische prognostische parameters. De vele gelijkenissen tussen de morfologische kenmerken van de bloed- en lymfevaten in de caniene melkkliertumoren met humane borsttumoren is een bijkomende reden voor het gebruik van honden met spontaan ontstane melkkliertumoren als diermodel in comparatieve oncologiestudies.

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Curriculum Vitae

Nathalie Sleeckx werd geboren op 6 maart 1983 te Lier. In 2001 beëindigde ze het secundair onderwijs, richting Moderne Talen-Wetenschappen, aan het Sint-Ursula lyceum in Lier en startte ze de studie Diergeneeskunde aan de Universiteit Antwerpen. Ze haalde in 2004 haar Bachelor diploma in de Diergeneeskunde met onderscheiding en verhuisde voor de Master opleiding naar de Universiteit Gent. In 2007 behaalde ze het diploma van Dierenarts (optie kleine huisdieren) met onderscheiding. Onmiddellijk na het afstuderen werd ze geselecteerd voor een Small Animal Rotating Internship aan de Universiteit Gent. Hierna was ze 6 maanden werkzaam in een tweedelijns dierenartsenpraktijk om dan begin 2009 terug te keren naar de Universiteit Antwerpen. Ze startte een doctoraatsonderzoek naar de angiogenese en lymfangiogenese in melkkliertumoren bij de hond. In 2013 vervolledigde ze haar doctoraatsopleiding. Nathalie Sleeckx is auteur en medeauteur van meerdere publicaties in internationale tijdschriften en nam actief deel aan nationale en internationale congressen.

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