Aus dem Institut für Tierpathologie

des Fachbereichs Veterinärmedizin der Freien Universität Berlin

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Circulating Tumor Cells as Indicators of Metastatic Spread of Canine Mammary Tumors

Inaugural-Dissertation

zur Erlangung des Doctor of Philosophy (Ph.D.)-Grades

in Biomedical Sciences

an der

Freien Universität Berlin

vorgelegt von

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Tierarzt aus Porto, Portugal

Berlin 2012

Journal-Nr.: 3619

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
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Deskriptoren (nach CAB-Thesaurus):
Dogs, mammary gland neoplasms, metastasis, circulation, tumor, cells, detection, reverse transcriptase PCR, messenger RNA, markers

Tag der Promotion: 30.05.2013

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-364-6
Zugl.: Berlin, Freie Univ., Diss., 2012
Dissertation, Freie Universität Berlin
D 188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

For my parents

Contents

List of Abbreviations

AgNORs	**Arg**yrophil Proteins associated with **N**ucleolar **O**rganizer **R**egions
AGR2	**A**nterior **Gr**adient **2** homolog
ATP8B1	**ATP**ase, Aminophospholipid Transporter, Class I, Type **8B**, Member **1**
BRCA1	**Br**east **Ca**ncer **1** early onset
BRCA2	**Br**east **Ca**ncer **2** early onset
CC	**C**arcinoma **C**ell
CK19	**C**yto**k**eratin 19
CLDN7	**Cl**au**din**-**7**
CMT	**C**anine **M**ammary **G**land **T**umors
CTC	**C**irculating **T**umor **C**ells
EGFR	**E**pidermal **G**rowth **F**actor **R**eceptor **1**
ELF3	**E**74-**l**ike **F**actor **3**
EpCAM	**Ep**ithelial **C**ell **A**dhesion **M**olecule
ER	**E**strogen **R**eceptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, also known as epidermal growth factor 2
F3	Coagulation **F**actor **III**, Tissue Factor, Thromboplastin
FGR1	**F**ibroblast **G**rowth **F**actor **R**eceptor 1
GHR	**G**rowth **H**ormone **R**eceptor
IC	Inflammatory Carcinoma
IGF1	**I**nsulin-like **G**rowth **F**actor **1**
IGF1R	**I**nsuline-like **G**rowth **F**actor **1 R**eceptor
IGF2	**I**nsulin-like **G**rowth **F**actor **2**
IGF2R	**I**nsuline-like **G**rowth **F**actor **2 R**eceptor
INSR	**Ins**ulin **R**eceptor
IRX3	**Ir**oquois Homeobo**x 3**
MIB1a	Mouse anti-Human Proliferation Antigen
MT	**M**ammary **G**land **T**umors

MVD	**M**icro**v**essel **D**ensity
p21	Cyclin-dependent Kinase Inhibitor 1A
p53	Tumor Suppressor Gene **p53**
PB	**P**eripheral **B**lood
PBL	**P**eripheral **B**lood **L**eukocytes
PCNA	**P**roliferating **C**ell **N**uclear **A**ntigen
PR	**P**rogesterone **R**eceptor
PTEN	**P**hosphatase and **Ten**sin homolog
SLC1A1	**S**o**l**ute **C**arrier Family **1**, member **1**
TGFBR	**T**ransforming **G**rowth **F**actor **B**eta **R**eceptor
WHO	**W**orld **H**ealth **O**rganization

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Introduction

1.1 Canine Mammary Gland Tumors

Cancer is the most common disease associated cause of death of dogs. It has been estimated that between 15 to 30 % of the dogs die with cancer (Bronden et al., 2010). Skin neoplasms are the most common canine tumors accounting for 43 % of the canine neoplasms, followed by mammary gland tumors (MT) with 28% (Bronden et al., 2010; Moulton, 1978). MT are the most common neoplasm of the female dog (representing 25 to 30 % of all tumors in the bitch) and approximately 50 % of them are histologically classified as malignant (Moulton, 1978; Lana et al., 2007). Canine mammary tumors (CMT) comprise a very heterogeneous group of tumors in terms of their morphology and biological behavior and share many similarities with the disease in humans (Nerurkar et al., 1989; Nieto et al., 2000).

Different studies propose a breed predisposition of developing MT for the boxer, cocker spaniel, English springer spaniels, dachshunds Toy and miniature poodles, Brittany spaniels, English setters, pointers, German shepherds, Beagle, Maltese and Yorkshire terriers (Boldizsar et al., 1992; Kurzman and Gilbertson, 1986; MacVean et al., 1978; Yamagami et al., 1996). Moreover, small breed dogs seem to be less predisposed to malignant MT than large breed dogs (Itoh et al., 2005). The median age of onset of MT tumor is 10 to 11 years, they rarely occur in animals under 4 years of age and youngers dogs are more likely to develop benign tumors than older dogs (Zatloukal et al., 2005). The risk of development of MT is significantly reduced with early ovariohysterectomy revealing a hormone dependent development of the tumors. The risk for developing malignant tumors in dogs neutered before the first estrus is 0.05 %; after the first estrus is 8 % and it increases to 26 % after the second estrus. No reduction on the risk for malignant tumors can be observed for dogs spayed at later time points (Schneider et al., 1969).

1.2 Diagnosis of CMT

The clinical diagnose of CMT generally involves collection of a complete medical history, physical examination with careful palpation of the mammary gland and hematologic and serum chemistry profiles (Lana et al., 2007). Additionally thoracic X-rays in three different planes and ultrasonographic evaluation may be performed to exclude lung and abdominal metastases. The most common sites of distant metastasis are the lungs, liver and less frequently bone (Lana et al., 2007). Fine needle aspirates of suspicious lymph nodes

combined with a cytological evaluation of the specimens to exclude possible lymph node involvement should also be performed. The most commonly affected lymph nodes besides the regional lymph nodes are the inguinal, sublumbar, sternal, and prescapular lymph nodes (Lana et al., 2007). In the dog MT may be presented as solitary or multiple nodules and may affect one or more mammary complexes (Misdorp, 1999; Lana et al, 2007). A recent study by Sorenmo and colleagues observed that 70 % of the dogs with MT have more than one tumor and that the two caudal pairs of gland are more frequently affected (Benjamin et al., 1999; Moulton et al., 1986; Sorenmo et al., 2009). Clinical features such as rapid growth, poor demarcation, superficial ulceration, lymph node enlargement, emaciation and dyspnea may suggest malignant behavior, whereas slow growth and good demarcation speak for benign neoplasias (Misdorp, 1999; Lana et al., 2007). Definitive diagnosis requires excisional biopsies of the tumor mass followed by histopathological examination which is considered the gold standard for CMT diagnosis (Sorenmo et al., 2011; Lana et al., 2007).

1.3 Prognosis of MT

Due to the high incidence of MT in the female the dog, in the past years there have been many attempts to predict post-surgical clinical outcome (Benjamin et al., 1999; Karayannopoulou et al., 2005). Because of the heterogeneity of biologic behaviors of CMT finding a classification system that provides suitable and relevant prognostic categorization is rather difficult (Dutra et al., 2004b). Several clinicopathological features have revealed prognostic significance and have been proposed as prognostic factors such as: clinical staging tumor size, lymph node status and distant metastasis, histological type, evidence of lymphoid cellular activity in the tumor vicinity, steroid receptor status, expression of oncogenes, tumor suppressors and adhesion molecules, proliferation markers and microvessel density among others. In the sections below the different prognostic factors and the different findings according to research groups are described in more detail.

1.3.1 Clinical Staging

CMT can be staged according to the TMN staging system either in its World Health Organization (WHO) original or modified form. Since the modified staging system is the most recent only the modified system will be addressed. The WHO TNM system gathers information about tumor size, lymph node status and presence of distant metastasis. Tumor size is assessed by measuring the greater diameter of the largest malignant tumor present. The presence of lymph node involvement or distant metastasis is performed as described

above. The tumors are then classified into stage I, II, III, IV and V (Table 1) (Lana et al., 2007).

Table 1 Modified WHO Staging System

Modified WHO Staging System

Stage	Tumor Size(T)	Regional Lymph node Status (N)	Distant Metastasis (M)
I	T_1	N_0	M_0
II	T_2	N_0	M_0
III	T_3	N_0	M_0
IV	any T	N_1	M_0
V	any T	any N	M_1

Tumor size (T): T_1 < 3 cm, T_2 3-5 cm, T_3 > 5 cm;

Regional lymph node status (N): N_0 no metastasis, N_1 metastasis;

Distant metastasis (M): M_0 present, M_1 absent

Clinical staging has proven prognostic significance in fact Chang and colleagues found significant prognostic differences in dogs with stage I, II and III MT when compared with stages IV and V(Chang et al., 2005). In another study Philibert and colleagues found that patients with stage I MT had a longer survival time than dogs with higher stages (Philibert et al., 2003).

1.3.2 Tumor Size

Tumor size is one of the features assed in tumor staging but there is a general agreement that size alone has prognostic significance, however discrepancies between different studies have been observed. In one study by Kurtzmann and colleagues dogs with T1 tumors had a better clinical outcome than dogs with T2 and T3 tumors, i.e. there was higher risk of recurrence in patients with tumors with a diameter greater than 3 cm but no significant differences was observed between T2 and T3 dogs (Kurzman and Gilbertson, 1986). In another study by Yamagami and colleagues T1 and T2 dogs had similar prognosis but T3 dogs had significant worst prognosis (Yamagami et al., 1996). Chang and colleagues demonstrated that dogs with tumors larger than 5 cm had worst survivals than those with tumors smaller than 5 cm (Chang et al., 2005). Thus there seems to be a difficulty in defining at which size the prognosis changes, the common notion that size has prognostic significance prevails (Sorenmo et al., 2011).

1.3.3 Lymph node status and metastasis

Lymph node involvement was related with poorer disease-free survival and recurrence after 6 months in 80% of the cases (Gilbertson et al., 1983; Kurzman and Gilbertson, 1986; Yamagami et al., 1996). On the other half dog patients without lymph node involvement had a recurrence rate of 30 % or lower within two years after surgery.(Kurzman and Gilbertson, 1986) In another study by Karayannopoulou and colleagues with a two year clinical follow-up 86 % of the patients with lymph node involvement died of disease contrasting with the 21 % that had negative lymph nodes (Karayannopoulou et al., 2005). The presence of distant metastasis is also associated with a poorer prognosis as was demonstrated by Philibert and colleagues in which the median postoperative survival for patients with distant metastasis was only 5 months whereas for patients without metastasis it was 28 months at presentation (Philibert et al., 2003).

1.3.4 Histological Type

According to the WHO malignant CMT can be grossly divided in those of mesenchymal origin or sarcomas and those of epithelial origin or carcinomas. Sarcomas include fibrosarcoma, osteosarcoma, carcinosarcoma, i.e. composed by cells that resemble epithelial and fibrous connective tissue components, carcinoma or sarcoma in benign tumor and other types of sarcomas. Carcinomas include carcinoma in situ, i.e. malignant epithelial tumors with no invasion of the basement membrane, complex carcinomas, simple carcinomas and special types of carcinomas such as spindle cell carcinomas, squamous cell carcinomas, mucinous carcinomas and lipid-rich carcinomas. Simple carcinomas are then further divided in tubulopapillary, solid and anaplastic carcinomas. This subdivision of simple carcinomas is believed to reflect the potential for malignancy (Lana et al., 2007). Benign tumors include adenoma, simple, complex and basaloid fibroadenoma, benign mixed tumor and duct papilloma (Misdorp et al., 1999).

Sarcomas are thought to have a poor prognosis and most of the dogs die within 9 to 12 months (Hellmen et al., 1993; Misdorp et al., 1971). For carcinomas the different histological subtypes have demonstrated different median survivals and therefore different prognosis (Philibert et al., 2003). Dogs with anaplastic carcinomas seem to have worst postoperative prognosis than dogs with adenocarcinoma, solid adenocarcinoma and other types of tumors. More precisely, in one study it was demonstrated that dogs with anaplastic carcinoma had a median survival of 2.5 months whereas dogs with adenocarcinoma, solid carcinoma and other tumor types had a higher median survival of 21, 16 and 24 months respectively (Philibert et al., 2003). Inflammatory carcinomas (IC) are difficult to resect surgically and

sometimes the resection of the tumor may not be possible (Lana et al., 2007) In cases where resection is possible these tumor tend to recur one month after surgery and in one study affected dogs had a mean survival of 25 days, therefore are considered to have poor prognosis (Alenza et al., 2001). Another study reported that 81 % of dogs with IC had distant metastases and 5 % regional metastases and the median survival was 60 days (Marconato et al., 2009).

1.3.5 Histological Grade

In the Elston / Ellis grading system the level of differentiation of tumors is assessed. In this grading system tumors are classified according to features such as tubular formation, nuclear pleomorphism and mitotic counts. Each feature is the scored from 1 to 3 and scores for each individual feature are added to obtain a final sore which corresponds to the grade: grade I or well differentiated (3-5 points), grade II or moderately differentiated (6-7 points) and grade III or poorly differentiated (8-9 points) (Table 2)

In one study Karayannopoulou and colleagues demonstrated lower survivals for dogs with grade III tumors when compared with dogs with grade II and I dog tumors. Dogs with undifferentiated tumors (grade III) also had higher risk of death when compares with dogs with more differentiated tumor (grade II and I) hence the author concluded that this grading system that was originally conceived for breast cancer (BC) had also prognostic significance for CMT (Karayannopoulou et al., 2005). In another study by Gilbertson and colleagues dogs with poorly differentiated tumors the risk of development of recurrence or metastatic disease was 90 %, whereas in moderately and well-differentiated tumors the risk was 68 % and 24 % respectively (Gilbertson et al., 1983).

Table 2 Elston & Ellis histological grading system

Tubule formation	Score	Mitotic count per 10 HPF	Score	Nuclear pleomorphism	Score
Most of the tumor (>75 %)	1	0 -9	1	Low	1
Moderate (10-75 %)	2	10-9	2	Moderate	2
Little or absent (0-10 %)	3	> 10	3	High	3

Grade	Final score
I (well differentiated)	3 -5
II (moderately differentiated)	6 - 7
III (poorly differentiated)	8 - 9

1.3.6 Lymphoid Reaction

Lymphocytic reaction in vicinity of the primary tumor is thought to be an inflammatory reaction against the tumor and it has also been correlated with prognosis. In the same study by Gilbertson and colleagues, dogs with grade I tumors without lymphocytic reaction were at higher risk of recurrence within two years, with a recurrence rate of 83%, than those with lymphocytic reaction that had a recurrence rate of only 45 % (Gilbertson et al., 1983).

1.3.7 Steroid Receptor Status

Ovarian steroid hormones play a role in the development of CMT as is demonstrated by the protective effects of ovariohysterectomy as previously referred (Donnay et al., 1996; Schneider et al., 1969). The expression of steroid receptors such as estrogen receptor (ER) and progesterone receptor (PR) in MT has also been correlated with prognosis. Nevertheless there are discrepancies on the prognostic relevance of the steroid receptor status between studies. In one study by Chang and colleagues, ER and PR expression was found in most benign neoplasias of the mammary gland of 95.8 % and 100 %) respectively (Chang et al., 2009). However, their expression decreased significantly in malignant tumors of 50.6 and 71.9 % respectively (Chang et al., 2009). The expression of these steroid receptors seemed also to be higher in tumors smaller than 5 cm which are considered to have better prognosis and also in those without lymph node and distant metastasis (Chang et al., 2009). Additionally, malignant tumors with expression of both ER and PR had a higher survival rate than those expressing only ER (Chang et al., 2009). Furthermore, PR expression was associated with one year survival after tumor resection, therefore PR expression was considered to be a prognostic factor for survival, especially in those malignant tumors with ER expression (Chang et al., 2009). Milanta and colleagues found a higher expression of ER in healthy tissues, hyperplasticand dysplastic lesions, and benign tumors than in carcinomas, suggesting a loss of ER expression in the malignant progression (Millanta et al., 2005). PR expression increased in dysplastic lesions and in carcinomas "in situ" and decreased in invasive carcinomas (Millanta et al., 2005). However, steroid receptor status and histological parameters and survival were not correlated (Millanta et al., 2005).

1.3.8 Expression of Oncogenes

The expression of several growth promoting genes such as epidermal growth factor 2 (ERBB2), epidermal growth factor 1 (EGFR1), transforming growth factor beta receptors (TGFBR), fibroblast growth factor receptor (FGFR), growth hormone receptor (GHR) and

insulin-like growth factor 1 and 2 receptors (IGF1R, IGF2R) and insulin receptor (INSR) have been associated with prognosis. However, their role and impact on the pathogenesis and prognostic significance in CMT is not yet fully understood.

ERBB2 is a tyrosine kinase receptor and is a member of the human epidermal growth factor receptor (HER) family. ERBB2 overexpression is found in 20 – 30% of breast cancers. Patients with ERBB2 overexpression usually have lower disease-free and overall survivals and therefore a poorer prognosis (Revillion et al., 1998; Slamon et al., 1987) (Ross et al., 2003). Due to its importance in BC, several groups tried to transpose the observations in women to the dog (Ahern et al., 1996; Dutra et al., 2004a; Hsu et al., 2009; Martin de las Mulas et al., 2003b). However, the findings contradicted those in BC. Hsu and colleagues reported ERBB2 overexpression in 29.7 % of canine malignant mammary tumors and observed that these dogs had a higher survival rate than those that expressed ERBB2 at a normal level (Hsu et al., 2009). Ahern and colleagues correlated ERBB2 overexpression with histopathologic diagnosis of malignancy but not invasiveness or regional metastatic disease (Ahern et al., 1996). Gama and colleagues also observed that dogs with ERBB2 overexpressing tumors have higher survival rates, but ERBB2 expression when combined with ER negativity and expression of basal cell markers such as P-cadherin, tumor protein p63, and Cytokeratin 5 was associated with poor prognosis (Gama et al., 2008a).

EGFR1 is also a tyrosine kinase receptor and member of the human epidermal growth factor receptor (HER) family. EGFR1 is overexpressed in 6 to 48 % of human BC and EGFR1 overexpression has been associated with poor clinical outcome (Tashiro et al., 2005; Toi et al., 1994; Tsutsui et al., 2002). In one study by Gama and colleagues, EGFR1 overexpression was found in 42.2 % of malignant CMT and its overexpression was also correlated with malignancy and lower overall survival, although no statistical significance was achieved (Gama et al., 2009).

Downregulation of several transmembrane growth factor receptors such transforming growth factor beta receptor (TGFBR), fibroblast growth receptor 1 (FGR1) growth hormone receptor (GHR) was found through transcriptome analysis in lymph node positive canine carcinomas when compared with normal mammary gland by Klopfleisch and colleagues, but no correlations with survival were yet established (Klopfleisch et al., 2011a; Klopfleisch et al., 2010c; Klopfleisch et al., 2011b).

The insulin-like growth factor 1 (IGF1) pathway is a high complex and regulated system that is important in human growth and in the development of several human cancers (Pollak, 2008). During carcinogenesis multiple components of this system become deregulated and provide growth and survival advantages to tumor cells (Hankinson et al., 1998; Otani et al., 2007). IGF-1 and insulin-like growth factor 2 (IGF-2) are ligands to the tyrosine kinases

receptors, insulin-like growth factor I receptor (IGF-1R) and the insulin receptor (INSR).and their binding provides cells proliferative and pro-survival signaling (Frasca et al., 1999). In CMT the IGF-1 pathway is believed to have similar impact. Klopfleisch and colleagues found that IGF1R, insulin-like growth factor II receptor (IGF2R) and INSR expression is unaltered in metastatic carcinomas (Klopfleisch et al., 2010a). Furthermore, IGF1 and IGF2 mRNA expression is decreased in malignant tumors (Klopfleisch et al., 2010a).

1.3.9 Tumor Suppressors

p53 tumor suppressor gene (p53) is involved in the carcinogenesis of human and canine cancers (Bergh et al., 1995; Lee et al., 2004). p53 mutations are the most common genetic alterations in CMT and several studies related p53 gene mutation with progression (Lee and Kweon, 2002; Mayr et al., 1998). p53 overexpression was considered by Lee and colleagues an independent factor for indicator of worse prognosis (Lee et al., 2004). Moreover, p53 gene mutations and protein were predictors of increased malignant potential and poor prognosis in CMT (Lee et al., 2004). However, in another study by Klopfleisch and colleagues found no significant p53 expression differences between adenomas, carcinomas and lymph node metastases. Furthermore, the same authors compared the expression of cyclin-dependent kinase inhibitor 1A (p21, another tumor suppressor) between adenocarcinomas and their metastases and observed a significant decrease in expression in metastases. The role of the phosphatase and tensin homolog (PTEN tumor suppressor) in the development of CMT was investigated by two work groups that had concordant observations. Lower expression of PTEN was found in malignant tumors and in the lymph node metastases, suggesting that lower PTEN expressions could play role in carcinogenesis and progression of CMT and also impact on prognosis (Kanae et al., 2006; Qiu et al., 2008).

The breast cancer 1 and 2 early onset genes (BRCA1 and BRCA2) are thought to be tumor suppressor genes and mutations of these genes were associated with BC susceptibility. These mutations are thought to lead to reduced DNA damage repair capability leading to damage accumulation thus increasing tumor development risk (Rivera et al., 2009; Wooster et al., 1995; Wooster et al., 1994). They are essential for DNA damage signaling and DNA repair. These tasks are mainly accomplished by the interaction and activation of DNA repair proteins such as RAD51 (Scully et al., 1997). Overexpression of BRCA2 and RAD51 is associated with a poor prognosis, whereas BRCA1 expression is often decreased during progression of sporadic breast cancer (Bieche et al., 1999; Maacke et al., 2000; Thompson et al., 1995). Rivera and colleagues associated BRCA1 and BRCA2 mutations with increased risk of MT in a dog breed (Rivera et al., 2009). Nieto and colleagues observed a

decrease in BRCA1 in malignant tumors (Nieto et al., 2003). Furthermore, Klopfleisch and colleagues observed that BRCA1 expression was not associated with histologic criteria of malignancy nevertheless, increased BRCA2 and RAD51 were found in metastatic tumors (Klopfleisch and Gruber, 2009).

1.3.10 Cell Adhesion Markers

Cadherins are adhesion molecules responsible for cell-cell interactions and have been implicated in the development of cancer. E-cadherin is a very widely studied cadherin and its downregulation was associated with tumor development and progression in BC (Birchmeier and Behrens, 1994; Zschiesche et al., 1997). In CMT progressive decrease of E-cadherin is observed in less differentiated malignant tumors (Restucci et al., 2007). Gama and colleagues reported a relationship between reduced membranous expression of E-cadherin with histological type, poor differentiation, high invasiveness, high proliferation index, lymph node metastasis and poor prognosis (Gama et al., 2008b).

1.3.11 Cell Proliferation Markers

Cell proliferation has been used in BC for evaluating biological behavior and malignancy potential (Hall and Levison, 1990). Ki67, proliferating cell nuclear antigen (PCNA), mouse anti-human proliferation antigen (MIB1a) are specific cell proliferation markers and allow the identification of dividing cells and the determination of the proliferation index by immunohistochemistry. Pena and colleagues identified a correlation of the PCNA index with malignancy and nuclear grades. Additionally, high index values of Ki-67 were positively correlated with metastasis, death from neoplasia and with low disease-free and overall survival. However the PCNA index failed to show significant association with these variables (Pena et al., 1998). The quantification of argyrophil proteins associated with nucleolar organizer regions (AgNORs) allows the evaluation of proliferative activity in situ. In one study by Sarli and colleagues, AgNOR index and MIB1a index was considered very useful for predicting the clinical outcome, i.e of tumors showing a higher AgNOR and MIB1a index shown a more aggressive behavior (Sarli et al., 2002).

1.3.12 Microvessel Density

Microvessel density (MVD) measures the blood supply of tumors which is believed to have impact on tumor behavior. MVD is visualized through immunohistochemistry for endothelial

cell markers. Studies observed that MVD count was significantly lower in benign tumor when compared with malignant. Additionally MVD is correlated with histological type and tumor grade and further, higher MVD counts are also correlated with poorer survival (Graham and Myers, 1999; Millanta et al., 2006; Restucci et al., 2000).

As described above several tumor features and several molecular markers have been used in attempt to predict the biological behavior of CMT. However discrepancies in the findings for the same factor are frequently observed in different research groups and so their relevance is therefore in many cases questionable (Matos et al., 2012). Moreover, the results between the research groups are difficult to compare due to lack of standardization. In a recent article by de Matos and colleagues the necessity of standardization of prognostic studies in CMT has been pointed out. In this publication the authors refer a necessity of uniformization of prognostic studies in terms of the methodologies and follow-up periods used (Matos et al., 2012). More importantly, prognostic factors for the occurrence of metastases which is a key point on tumor progression and has a major impact on survival are still lacking.

1.4 Metastasis

In women as well as in the female dog metastasis is the most frequent tumor-related cause of death (Jemal et al., 2008; Misdorp and Hart, 1976). The metastatic cascade is a very complex sequence of events that involves the completion of several steps. These include shedding of cells from the primary tumor into the circulatory system, survival of the tumor cells in circulation, arrest at a new site, extravasation, initiation and maintenance of growth and at last vascularization that allows the growth of the metastatic tumor at its new location (Chambers et al., 2002; Jiang et al., 2002a; Pantel and Brakenhoff, 2004). Although metastasis is widely studied process the exact events which are involved are far from fully understood.

During the tumor development it is believed that subsets of tumor cells within the primary tumors that possess numerous genetic abnormalities and display unregulated growth, at some point gain invasiveness and motility features that allow them to gain entrance in the circulation (Bednarz-Knoll et al., 2011; Hart et al., 1989; Jiang et al., 2002a). In BC these cells might enter the lymphatic vessels which lead to the draining lymph node where they may develop lymph node metastasis (Chambers et al., 2002). However, the most common sites for distant metastasis in breast cancer are the lungs, brain, liver and bone to where there are no direct lymphatic routes (Chambers et al., 2002). This fact lead to the belief that at some point tumor cells need to gain asses to the blood circulation in order to reach these

locations (Chambers et al., 2002). This might occur through the efferent lymphatic vessels that flow in to the venous circulation via thoracic duct of through newly formed blood vessels in the lymph node metastasis (Chambers et al., 2002; Sleeman et al., 2011). Tumor cells can also enter the blood circulation directly, bypassing the lymph nodes.(Pantel and Brakenhoff, 2004) In the vascular compartment this circulating tumor cells (CTC) travel to distant organs where they might settle on the vascular endothelium, extravasate in to the surrounding tissue, initiate growth and angiogenesis that will finally lead to the development and growth of a distant metastasis (Folkman, 1990; Jiang et al., 2002a). (Figure 1)

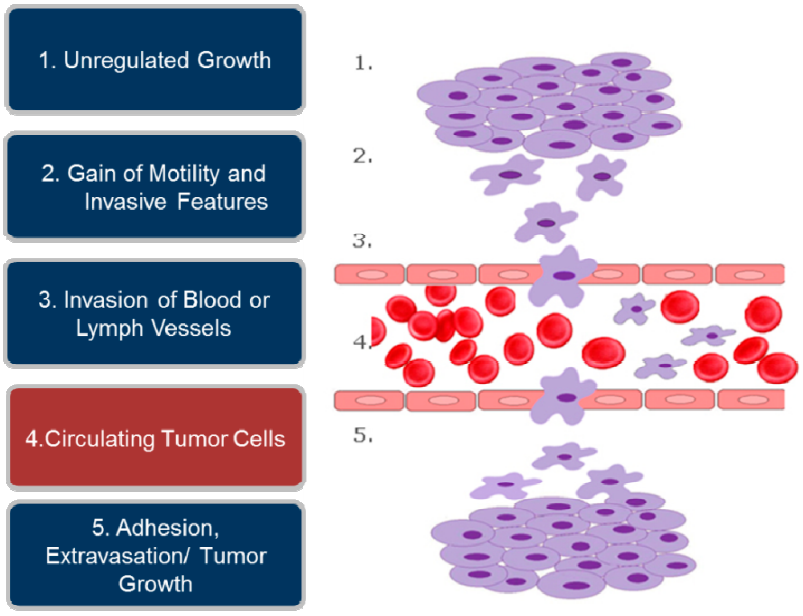

Figure 1 The metastatic cascade: sequence of events

1.5 Circulating Tumor Cells

CTC are tumor cells of epithelial origin present in the blood shed either from the primary tumor or its metastasis and that possess antigenic and/or genetic properties of a specific tumor type. CTC are found in the peripheral blood (PB) of patients with different types of carcinomas but are not present in patients with benign disease (Cristofanilli, 2006; Harris et al., 2007; Mego et al., 2010). This subset of tumor cells was first identified by Ashworth in 1869 and is considered rare among the numerous cells in the PB, occurring in numbers as low as 1 CTC per 10^5 to 10^7 peripheral blood leukocytes (PBL) (Ashworth, 1869; Mego et al., 2010).

1.5.1 CTC Detection Methods

Due to that fact that CTC are rare events, several detection and isolation methods for CTC have been developed. They grossly include cytometric and nucleic-acid based techniques which are usually preceded by an enrichment technique to improve sensitivity to an appropriate level (Mostert et al., 2009).

1.5.1.1 CTC Enrichment

Enrichment techniques may rely on physical CTC properties such as cell size and density or expression of tumor or epithelial surface markers (Mostert et al., 2009). Enrichment based on cell size uses filtration based on the assumption that CTC are larger than leukocytes.(Vona et al., 2000) Density-gradients have also been used and separate CTC and granulocytes from mononuclear cells which have lower densities (Vona et al., 2000). Finally, immunomagnetic separation can be performed using immunomagnetic beads labeled with monoclonal antibodies. CD45 for example is a panleukocyte marker used for negative selection of CTC (Jacob et al., 2007). Positive selection can be performed using anti-epithelial antibodies such as epithelial cell adhesion molecule (EpCAM) or tumor-specific antigens such as ERBB2 and mucin 1 (MUC1) (Allard et al., 2004; Mostert et al., 2009).

1.5.1.2 Detection Step

As stated previously detection methods include cytometric and nucleic-acid based methods. Cytometric methods isolate and innumerate individual CTC based on their antigen expression. The major advantage of this method is that it allows further characterization of cells. The major disadvantage on this approach is the lack of specific antibodies for instance for canine tumor cells (Mostert et al., 2009). The most widely used methods are RT-PCR and qRT-PCR targeting tumor and epithelial-specific markers such as ERBB2, cytokeratin 19 (CK19), Mammaglobin, MUC1, EGFR, EpCAM among others (Bosma et al., 2002; Fehm et al., 2007; Gradilone et al., 2003; Lin et al., 2003; Weigelt et al., 2003; Wulf et al., 1997). Nucleic-acid methods are broadly used and generally show high sensitivities, but low specificity due to the possible expression of markers in normal cells and therefore it is sometimes hard to distinguish a true positive signal. Like in cytometric methods there is also lack of specific markers for canine tumor cells (Mostert et al., 2009). The specificity may be improved with the introduction of a multimarker assays combining several breast cancer specific markers (Mostert et al., 2009).

1.5.2 CTC Detection in Breast Cancer: Clinical Relevance

CTC were the subject of intense study in recent years and their detection proven clinical usefulness in many types of cancers such breast (Cristofanilli, 2006; Hayes et al., 2006; Weigelt et al., 2003), lung (Krebs et al., 2012; Naito et al., 2012; Nieva et al., 2012), colorectal (Cohen, 2009; Molnar et al., 2008), gastric (Cao et al., 2011), pancreatic (de Albuquerque et al., 2012; Khoja et al., 2012), prostatic (de Bono et al., 2009; Saad and Pantel, 2012) and ovarian cancers (Poveda et al., 2011) as well as in melanoma (Schuster et al., 2011).

The clinical relevance of CTC has been more frequently studied in breast cancer patients probably due to the high incidence of the disease. In a retrospective study involving 151 BC patients using the CTC detection system CellSearch^{TM} , Cristofanilli and colleagues considered CTC an independent prognostic factor and demonstrated that patients with less than 5 CTC per 7.5 ml PBL had a median overall survival of 29.3 months and patients with 5 or more CTC had a significantly lower overall survival of just 13.5 months (Cristofanilli, 2006). Moreover patients with more than 5 CTC were at higher risk of death (Cristofanilli, 2006). In another study by Hayes and colleagues, 177 BC patients were evaluated before the initiation of a new course of therapy and several weeks after the initiation of therapy using the same CTC isolation system. From their observations the authors concluded that the detection of elevated CTCs at any time during therapy is an accurate indication of subsequent rapid disease progression and mortality for BC patients (Hayes et al., 2006). Furthermore in a study by Budd and colleagues, CTC detection through the CellSearch^{TM} system was considered an earlier, more reproducible indication of disease status than current imaging methods and that CTC correlate better with overall survival than do changes determined by traditional radiology (Budd et al., 2006). Pachmann and colleagues in a study using 91 non-metastatic primary BC patients, CTC were quantified using laser scanning cytometry of anti-epithelial cell adhesion molecule-stained epithelial cells from whole unseparated blood before and during adjuvant chemotherapy proved that CTC could be detected in all patients and that a 10-fold increase in CTC numbers in the PB between before and after chemotherapy was an independent predictor of disease relapse (Budd et al., 2006).

Regardless of the difference in the detection methods and detection rates, CTC detection has proven clinical relevance in patient with metastatic breast cancer and can help to predict their biological and clinical behavior in patients with BC.

1.6 Aims and Hypotheses

CTC and their clinical and prognostic relevance not only in breast but also in several other types of cancer have been broadly studied in human oncology in the past years. However this research field remains unknown in veterinary oncology. Based on the promising findings in BC research and the recognition of CTC detection as a powerful tool in the determination of risk, prognosis and response to treatment it seems possible that CTC detection might also reveal the same clinical usefulness and therefore this project was based in the following two major hypotheses.

1.6.1 First Hypothesis

Tumor cells are shed in dog patients baring malignant mammary gland tumors either from the primary tumors or its metastases into to the blood stream directly or indirectly via lymphatic circulation. Similarly to human breast CTC, canine mammary gland CTC occur at very low numbers but despite their rarity they should be detectable through highly sensitive nucleic-acid based methods such as RT-PCR using appropriate mRNA markers.

1.6.2 Second Hypothesis

The detection of CTC mRNA markers correlates with the histological evidence of vascular invasion by tumor cells in the vicinity of the primary tumor. CTC detection in dogs may also provide important and relevant clinical information in terms of prognosis.

Based on these conducting premises the aims of our project were the following:

1.6.3 First Objective

The identification of sensitive and specific mRNA for the detection of canine MT CTC following three different approaches: First, by evaluation of the mRNA expression of genes with high expression in CMT, second genes which are CTC markers for the detection of human breast cancer CTC, and third by genes which can be identified by comparison of the transcriptome of CMT with the transcriptome of peripheral blood leukocytes.

1.6.4 Second Objective

The presence of canine CTC markers in the peripheral blood correlates with the histological evidence of vascular invasion in the primary tumor.

Research Publications in Journals with Peer-Review

2.1 Potential markers for detection of circulating canine mammary tumor cells in the peripheral blood

"Potential markers for detection of circulating canine mammary tumor cells in the peripheral blood"

Vet J 2011;190:165-168, doi:10.1016/j.tvjl

http://dx.doi.org/10.1016/j.tvjl.2010.09.027

2.2 Identification of Six Potential Markers for the Detection of Circulating Canine Mammary Tumor Cells in the Peripheral Blood Identified by Microarray Analysis

"Identification of Six Potential Markers for the Detection of Circulating Canine Mammary Tumor Cells in the Peripheral Blood Identified by Microarray Analysis"

J Comp Pathol. 2012 Feb-Apr;146(2-3):143-51. doi:10.1016/j.jcpa.2011.06.004
http://dx.doi.org/10.1016/j.jcpa.2011.06.004

2.3 Multiple RT-PCR Markers for the Detection of Circulating Tumour Cells of Metastatic Canine Mammary Tumours

"Multiple RT-PCR markers for the detection of circulating tumour cells of metastatic canine mammary tumours."
Vet J. 2012 Oct 1. pii: S1090-0233(12)00370-X. doi: 10.1016/j.tvjl.2012.08.021.
http://dx.doi.org/10.1016/j.tvjl.2012.08.021

Declaration of Own Portions of Work in the Research Publications

3.1 Potential markers for detection of circulating canine mammary tumor cells in the peripheral blood

Authors: da Costa, A., Oliveira J.T., Gärtner, F., Kohn, B., Gruber, A.D., Klopfleisch, R.

Year: 2011

Journal: The Veterinary Journal, 190:165-168

Contributions by da Costa A.: Independent design, preparation, completion, evaluation and interpretation of all experiments including preparation of tissue samples, cell culture, RT-PCR, preparation of the manuscript.

Contributions of other authors: Development of the cell lines used as models (CMM26 and CMM115), collection of samples, development of clinical histories and histological diagnoses. Design, preparation and evaluation of results and preparation of the manuscript.

3.2 Identification of Six Potential Markers for the Detection of Circulating Canine Mammary Tumor Cells in the Peripheral Blood Identified by Microarray Analysis

Authors: da Costa A., Lenze, D., Hummel, M., Kohn, B., Gruber, A.D., Klopfleisch, R.

Year: 2012

Journal: Journal of Comparative Pathology, Feb-Apr;146(2-3):143-51

Contributions by da Costa A.: Independent design, preparation, completion, evaluation and interpretation of all experiments including preparation of tissue samples, cell culture, RT-PCR. Subsequent preparation of the manuscript except the part concerning the microarrays.

Contributions of other authors: Collection of samples, development of clinical histories, histological diagnoses and conduction of the microarrays. Design, preparation and evaluation of results and support of the preparation of the manuscript.

3.3 Multiple RT-PCR Markers for the Detection of Circulating Tumour Cells of Metastatic Canine Mammary Tumours

Authors: da Costa A., Kohn, B., Gruber, A.D., Klopfleisch, R.

Year: 2012

Journal: The Veterinary Journal

Contributions by da Costa A.: Independent design, preparation, completion, evaluation and interpretation of all experiments including preparation of tissue samples, cell culture, RT-PCR. Subsequent preparation of the manuscript.

Contributions of other authors: Collection of samples, development of clinical histories and histological diagnoses. Design, preparation and evaluation of results and preparation of the manuscript.

Concluding Discussion

Circulating tumor cells (CTC) have been the subject of attention of several research groups in the past years. In fact, detection of higher numbers aof CTC before and after initiation of chemotherapy is highly correlated with decreased overall and progression-free survivals and thus a powerful tool in determining prognostic and response to treatment (Cristofanilli, 2006; Hayes et al., 2006). However, CTC are extremely rare events, occurring in numbers of 1 CTC per 10^5 to 10^7 peripheral blood leukocytes (PBL) and therefore highly sensitive molecular methods such as RT-PCR or enrichments steps with immunomagnetic enrichment are required for their detection (Mego et al., 2010; Mostert et al., 2009).

On the other hand, in veterinary medicine studies regarding CTC were still lacking, there were no known canine-specific CTC markers and their numbers are still unknown.

4.1 mRNA Marker Identification and in-vitro Sensitivities

The aim of the first part of the project was the identification of potential mRNA markers for CTC detection through RT-PCR following three different approaches. The first approach consisted in testing known human breast CTC mRNA markers (approach 1). Second genes known to be overexpressed in CMT were tested (approach 2). Third microarrays were performed to compare the differences between the expression patterns of PB cells and cultivated canine malignant MT cells from cell lines CMM26 and CMM115 in order to find further canine-specific CTC markers (approach 3). Combining these three different approaches several genes were identified and from those PCR assays were established. These were than tested for their expression on peripheral blood (PB) samples of healthy female dog donors (n=10), tissue samples of metastatic adenocarcinomas (n=10) and two malignant CMT cell lines (CMM26 and CMM115). The selection criterion for the potential markers was that they could not be expressed in the PB of the healthy donors and should be expressed in metastatic carcinomas and malignant cell lines (Figure 2). Following this selection criterion 12 potential markers were identified, six from our two first approaches (CK19, EGFR, ERBB2, CLDN7, ELF3 and maspin) and six from our third approach (AGR2, ATP8B1, CRYAB, F3, IRX3 and SLC1A1) were considered specific genetic markers for CMT CTC.

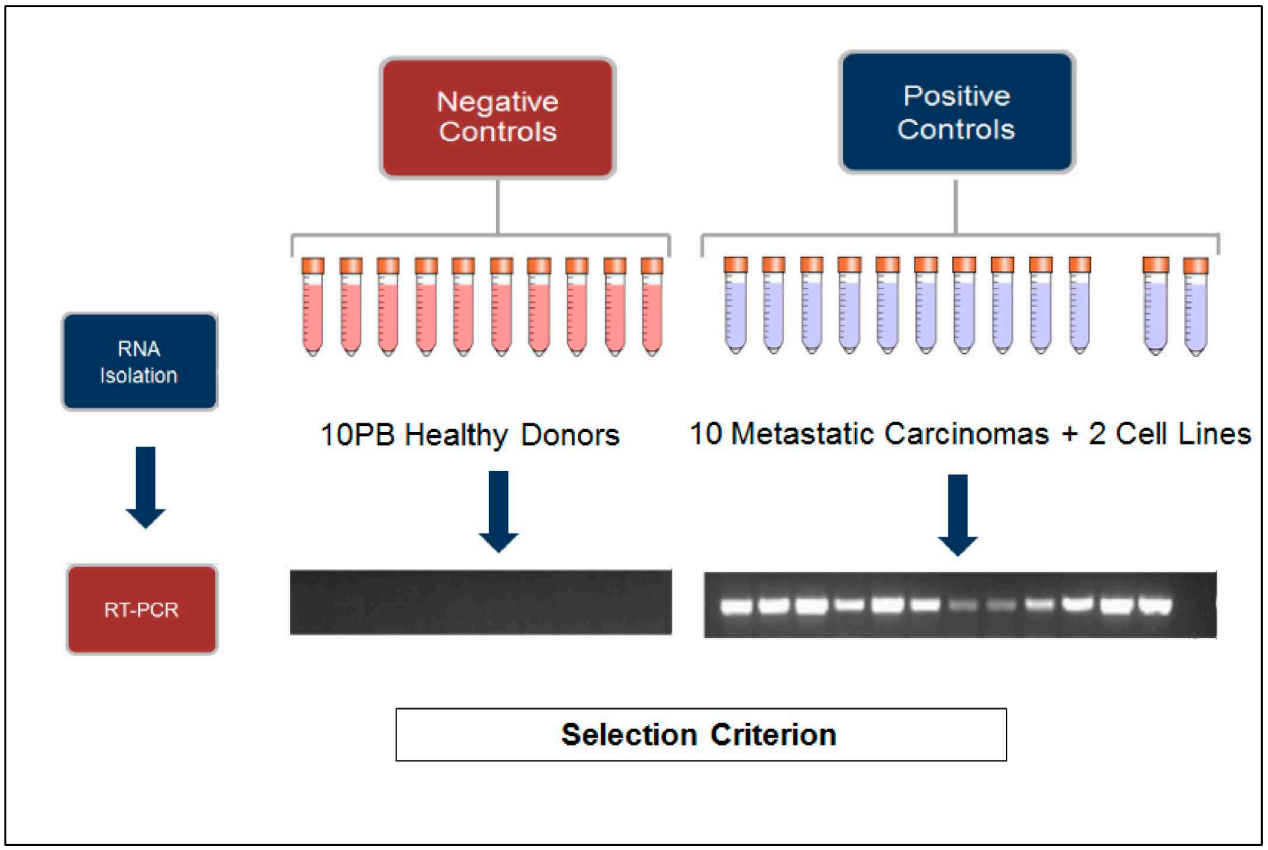

Figure 2 Schematic representation of the mRNA marker selection process.

For the mRNA marker selection markers were tested through RT- PCR with peripheral blood samples (PB) of healthy dogs, ten metastatic carcinomas and two cell lines. Selection criterion was: no expression in peripheral blood (PB) and expression in carcinomas and cell lines.

In a second step the sensitivity of the 12 RT-PCR markers was assessed under controlled conditions by testing the ability to detect very low numbers of cultivated tumor cells from cell lines CMM26 and CMM 115 admixed in PB samples of healthy donors (Figure 3). The required sensitivity level was chosen according to reports on CTC numbers in human BC patients and hence a target detection sensitivity of 1 CC per 10^6 to 10^7 PBL was established. From the 12 candidates, 11 successfully detected tumor cells within the desired sensitivity interval. Maspin was the only marker that failed to achieve the desired sensitivity (Figure 3). The ability of most markers to detect tumor cells at very low numbers suggested that they could also be able to detect CTC in the PB of dog patients with malignant MT. Although the sensitivity of most markers was within the desired interval, the introduction of an enrichment step before the detection step would possibly further raise the sensitivity of the assay. However, since the results at this point were promising and since the use of immunomagnetic enrichment devices could raise the costs of the study, it was decided not to include an enrichment step.

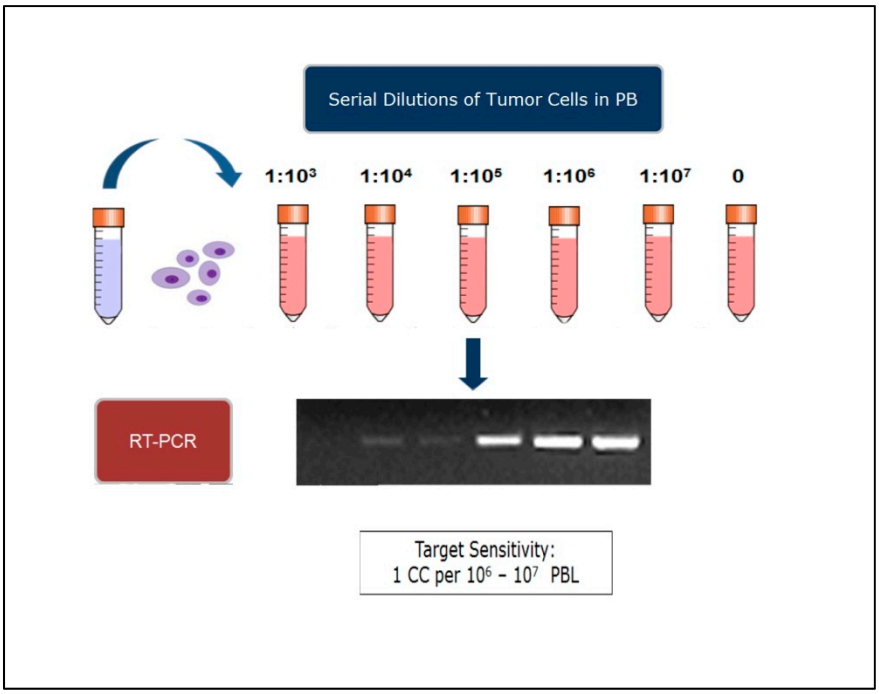

Figure 3 Schematic representation of the “in vitro” sensitivity testing.

The “in-vitro” sensitivity testing was performed by using serial dilutions of tumor cells in peripheral blood samples from healthy dogs. Target sensitivity for the candidate markers was 1 CC per 10^6 to 10^7 PBL

4.2 Identified CTC Markers

4.2.1 CK19

CK19 is an intermediate filament and one of the most widely used CTC markers, not only in breast cancer but also in several other tumor types (Bosma et al., 2002; Chen et al., 2007; Hardingham et al., 2000; Iinuma et al., 2006; Ikeguchi et al., 2003; Kahn et al., 2000; Winter et al., 2009). The broad use of CK19 as a CTC marker in human breast cancer is based on the fact that it is a pan-epithelial marker that is not expressed in normal lymphoid or hematopoietic tissues (Datta et al., 1994; Schoenfeld et al., 1994). The ability to sensitively detect a wide variety of epithelial cells by CK19 is obviously also a disadvantage of CK19. False-positive results in normal peripheral blood were attributed to contamination with epithelial cells from the skin during the blood withdrawals and the lack tumor specificity have been discussed widely (Mostert et al., 2009). Similarly, CK19 mRNA also appears to be sensitive marker for canine mammary tumor CTC being able to detect up to 1 carcinoma cell (CC) per 10^7 PBL showing the highest sensitivity in this group. However it can be expected that CK19 is not specific for CMT cells alone. Furthermore, widespread expression of CK19 requires appropriate blood withdrawal without contamination with skin epithelial cells.

4.2.2 CLDN7

Claudin 7 (CLDN7) is a member of the claudin family which are membrane proteins and components of tight junctions (Hewitt et al., 2006). CLDN7 is known to be over-expressed several human malignancies such as those originating from the pancreas, bladder, thyroid, fallopian tubes, ovary, stomach, colon, breast, uterus, and the prostate (Hewitt et al., 2006). In one study CLDN7 positivity was associated with reduced time of recurrence, suggesting a contribution of this marker to aggressiveness of breast cancer (Bernardi et al., 2012). Another study correlated CLDN7 expression loss with nodal metastasis (Kominsky et al., 2003). However in another study strong expression of CLDN7 in the metastatic lesions was correlated with the size of the largest metastatic focus. Furthermore up-regulation of CLDN7 was found in metastatic tumors, suggesting that CLDN7 expression might be necessary for facilitating metastasis (Park et al., 2007). This marker has also been used for CTC detection in patients with breast cancer due to its constant expression in breast cancer cells (Gervasoni et al., 2008). Expression studies of CLDN7 in CMT are still lacking, nevertheless in this study CLDN7 was considered a potential marker for CTC detection and was also shown a suitable maximum sensitivity of 1 CC in 10^7 PBL.

4.2.3 EGFR

As already discussed above EGFR plays an important role in cell regulation, proliferation and survival and EGFR overexpression has been associated with malignant behavior of canine mammary tumors (Gama et al., 2009; Salomon et al., 1995). Several studies refer to EGFR as a useful marker for CTC detection in human breast cancer and other cancer patients (Clark and Muchowski, 2000; De Luca et al., 2000; Gazzaniga et al., 2001; Raynor et al., 2002). However, one study found illegitimate expression in healthy blood donors using nested-PCR and southern blotting for colon cancer CTC detection (Raynor et al., 2002). In this study no illegitimate expression was found in healthy, canine, peripheral blood and EGFR was therefore considered as a potential marker of CMT CTC with a detection sensitivity of 1 CC in up to 10^7 PBL.

4.2.4 ELF3

E74-like factor 3, also known as ELF3 is a member of the Ets multigene family of transcriptional regulators that regulate several normal biologic activities including development, differentiation, homeostasis, proliferation and apoptosis (Chang et al., 1997; He et al., 2007). Its expression in humans is restricted to tissues of epithelial origin including

mammary gland epithelium (Chang et al., 1997; Kopp et al., 2007). ELF3 is also expressed at higher levels in breast cancer cells when compared with normal mammary gland cells (He et al., 2007). Loss of ELF3 activity leads to significant decreases in ERBB2 expression, suggesting an influence on ERBB2 expression (Clark and Muchowski, 2000). In this study ELF3 mRNA was not detected in healthy canine PB and it allowed the detection of 1 CC in up to 10^6 PBL.

4.2.5 ERBB2

As referred before ERBB2 is a member of the epidermal growth transmembrane receptor tyrosine kinase family (Slamon et al., 1987). ERBB2 signaling is involved in the induction of many types of human cancers due to its positive effect on proliferation and cell survival (Slamon et al., 1987). It is also known to be over expressed in 20-30% of human breast cancers and correlated with aggressive behavior and poor prognosis (Slamon et al., 1987). In canine mammary tumors ERBB2 is expressed in the majority of the carcinomas and overexpression is associated with poorer prognosis (Ahern et al., 1996; Martin de las Mulas et al., 2003a). ERBB2 is regarded a suitable and sensitive marker for human breast cancer CTC and has been widely used in human breast cancer CTC studies (Fonseca et al., 2002; Raynor et al., 2002). In the present study ERBB2 expression was not found in the PB of healthy dogs, was present in all carcinomas and cell lines and RT-PCR based detection shown a maximum sensitivity of 1 CC in 10^6 PBL.

4.2.6 Maspin

Maspin is a member of the serpin family protease inhibitors (Zhang, 2004; Zhang et al., 2000). It is believed that maspin acts as a tumor suppressor inhibiting motility, invasion and metastasis. Maspin showed impaired expression in several types of human carcinomas (Zhang, 2004; Zhang et al., 2000; Zou et al., 1994). Expression levels in epithelial and myoepithelial cells of CMT have been discussed controversially. While one study found an overexpression in malignant CMT other studies found no significant expression differences or decreased maspin expression in these tumors (Espinosa de los Monteros et al., 2005). The present study confirmed maspin as a potential CTC marker, however a poor sensitivity was observed for this particular marker (up to 1 CC per 10^4 PBL). This fact might be explained might due to the reported decreased maspin expression in malignant canine mammary tumor cells (Klopfleisch et al., 2010b). However, although downregulated in tumor

cells maspin is expressed in mammary tumors and relatively specific for mammary epithelium (Zou et al., 1994).

4.2.7 ATP8B1

The ATPase, aminophospholipid transporter, class I, type 8B, member 1 (ATP8B1), is a P4-type ATPase that is involved in the maintenance of cation gradient across cellular membranes (Kuhlbrandt et al., 1998). In humans ATP8B1 expression is thought to be restricted to several epithelial cell types including small intestine, pancreas, urinary bladder, stomach, prostate and liver (Bull et al., 1998; Dawson, 2010). More specifically in the liver, ATP8B1 is localized on the canalicular membrane of hepatocytes, but is also expressed at the apical membrane of polarized epithelial cells in many other tissues (Dawson, 2010). In this study ATP8B1 has shown high expression in CMT cells but not in PBL which are of mesenchymal origin. In addition, ATP8B1 mRNA expression levels seem to be consistently high in canine mammary carcinomas and allowed CTC detection with a high sensitivity for both cell lines being able to detect up to 1 CC per 10^7 PBL. Nevertheless, despite its high expression in CMT CTC, ATP8B1 may be expressed in other canine epithelial tissues and their tumors. Further studies are therefore needed to test whether ATP8B1 is exclusively expressed in CMT CTC or also in CTC of other canine epithelial tumors.

4.2.8 AGR2

The anterior gradient 2 homolog (AGR2) belongs to the anterior gradient family of protein and is overexpressed in several human epithelial malignancies such as breast, lung, small intestine, prostate, pancreas and esophagus cancers.(Fletcher et al., 2003; Fritzsche et al., 2007; Hao et al., 2006; Lowe et al., 2007; Thompson and Weigel, 1998). AGR2 is associated with estrogen receptor alpha activity and elevated AGR2 expression was detected in ERA-positive human breast cancer and correlated with poor prognosis (Hrstka et al., 2010; Thompson and Weigel, 1998). In the literature there are no expression studies concerning AGR2 in CMT. In this study AGR2 was specifically expressed in metastatic carcinomas and malignant mammary gland cell lines and not in the PB of healthy dogs, moreover it was able to identify up to one CC per 10^6 PBL.

4.2.9 CRYAB

Crystallin, alpha B (CRYAB) is a member of the heat shock protein family which promotes cell survival and protection against apoptotic stimuli (Kamradt et al., 2005). CRYAB is known to be expressed in human, basal-like breast carcinomas and is an independent predictor of poor survival of breast cancer patients (Moyano et al., 2006). Overexpression of CRYAB immortalizes human mammary epithelial cells and contributes to a more aggressive tumor cell behavior (Moyano et al., 2006). In the present study CRYAB proved to be a suitable marker for canine mammary CTC detection. In CMT higher levels of CRYAB expression were found in malignant MT when compared with benign neoplasias and normal mammary gland, suggesting that CRYAB may confer a survival stimulus to cancer cells.(Guvenc et al., 2012) CRYAB proved to be a potential CMT CTC marker and was able to detect one CC in 10^6 PBL.

4.2.10 F3

F3 commonly known as tissue factor or thromboplastin is an important coagulation factor required to initiate the extrinsic pathway of the coagulation cascade (Contrino et al., 1996; Rak et al., 2008). F3 is also essential for embryo development, maintenance of vascular integrity and tissue repair (Contrino et al., 1996; Rak et al., 2008). It is widely expressed on cells of extravascular compartments and initiates hemostasis upon tissue injury. F3 has gained attention in several very recent studies due to the fact that several cancer cells including those of the breast show aberrant high levels of F3 (Contrino et al., 1996; Rak et al., 2008). Cancer cells and their vascular stroma often show prothrombotic properties by for instance deregulation of F3 expression (Contrino et al., 1996; Rak et al., 2008). F3 has also been related with tumor progression by promoting tumor growth, angiogenesis, cell migration features which are necessary for the development of metastases (Contrino et al., 1996; Rak et al., 2008). In this study F3 was expressed in metastatic CMT and in malignant MT cell lines and was not expressed in PBL. Furthermore it was able to identify up to 1 CC per 10^6 PBL.

4.2.11 IRX3

The iroquois homeobox 3 of transcription factor (IRX3) plays an important role during embryonic development being able to act either as repressors or activators of gene expression (Gomez-Skarmeta and Modolell, 2002; Zhang et al., 2011). Until this moment there is no association between IRX3 expression and carcinogenesis. In the present study

IRX3 was considered a potential CTC marker because it full field our selection criterion and was sensitive enough to detect up to 1 CC per 10^6 PBL.

4.2.12 SLC1A1

SLC1A1 or solute carrier family 1, member 1 is a high-affinity anionic amino acid transporter and in human it is expressed in a wide variety of epithelial tissues, kidney, brain, and eye (Bailey et al., 2011). Similarly to IRX3, no association between carcinogenesis and its expression was found. This potential CTC marker was sensitive enough to detect up to 1 CC per 10^6 PBL.

Resuming the results from the first part of the project, 12 markers full field the selection criterion and were considered potential canine mammary CTC markers. The tissue expression studies of these markers, either in humans or in dogs, suggest that none of them would be specific for CMT CTC. From these 12, 11 also achieved the minimum target detection sensitivity of 1 CC per 10^6 PBL. Interestingly the sensitivities of the different markers varied according to the cell lines used in the sensitivity testing. CMT comprise a very heterogeneous group of tumors that often display different expression patterns. The differences in sensitivities may therefore possibly be caused by the differences in expression observed by the two cell lines used as tumor models in this study (CMM26 and CMM115). Furthermore, sensitivity testing of the markers was performed under controlled conditions using serial dilutions of CC in the peripheral blood of healthy donors and therefore the markers ability to identify CTC in real MT baring patients had to be determined.

4.3 CTC Detection in Dogs with mammary tumors

To determine the ability of the markers to detect CTC in female dogs with MT a total 120 PB samples from dog baring different types of MT were gathered with cooperation of several small animal clinics in Germany. The blood samples were then divided into three groups according to the histological diagnosis of the MT. Group 1 (n=40) included malignant mammary gland carcinomas with histological evidence of vascular invasion of tumor cells near the primary tumor or presence of metastatic cells in the regional lymph node, group 2 (n=40) included malignant mammary gland carcinomas without histological evidence of vascular invasion and metastasis free lymph nodes, and group 3 (n=40) included patients with benign mammary gland adenomas.

All the samples were then tested through RT-PCR with a panel of seven pre-selected potential CTC mRNA markers for the detection of their expression in PB of these patients. From the 12 selected markers maspin was excluded directly due to its lack of sensitivity of only 1 CC per 10^5 PBL. CK19 showed high sensitivity in the sensitivity testing, however when applied in this large scale assays several contamination problems were detected, namely positive negative controls were frequently observed and therefore it was excluded from the panel. IRX3 and ERBB2 were excluded from the panel due to technical problems related with the PCR efficiency that were not solvable. The last marker excluded was AGR2 because in the first round of tests it showed constantly low sensitivity and the RT-PCR assay failed to detect its expression in all 120 PB samples. Finally, the panel of markers used in this final phase was CLDN7, CRYAB, ELF3, SLC1A1, ATP8B1, EGFR and F3. Three rounds of tests were performed and a positive result was considered when positivity was observed in at least two of the rounds for each sample. After concluding the assays, the statistical sensitivity of the markers was calculated, i.e. the measure of the proportion of actual positives results which are correctly identified as such. This statistical measure should not be confused with the sensitivity assessed in the first part of the project which measured the ability of the markers to detect dilutions of CC cells in the PB of healthy donors. Another statistical parameter tested was the specificity, i.e. the proportion of negatives which are correctly identified. Finally the statistical significance of the differences in the numbers of positive PB samples between tumor groups was analyzed using the Kruskal–Wallis $p<0.05$ was considered statistically significant.

4.3.1 Sensitivity and Specificity of Individual Markers.

The sensitivity of the markers to detect CTC, i.e. the ability to detect potentially blood of dogs with metastatic tumors was moderate for all markers tested. CRYAB and ELF3 were the most sensitive markers with a sensitivity of 35 %.CRYAB showed a specificity of 100 % but in contrast, ELF3 had a specificity of 55 % only. ATP8B1 and EGFR showed sensitivities of 32.5 % and 27.5 % and where also highly specific with 90 % and 92.5 %. CLDN7, F3 and SLC1A1 had very low sensitivities. From these results CRYAB appears to be the most useful individual marker with the best combination of sensitivity and an ideal 100% specificity. Additionally, a statistical difference in the number of CRYAB- positive blood samples between group 1 and 2 and between group 2 and 3 was detected.

ELF3 although equally sensitive, shows a remarkably low specificity translated by the high number of positive results observed for group 2 and 3 and therefore may not be an adequate CTC marker. ATP8B1 and EGFR also had relatively promising performances both in terms of

sensitivity and specificity and therefore could also be considered as possible individual CTC markers. Nevertheless, it was also necessary to verify if combining the detection abilities of individual markers in multimarker assays could overcome the detection performances of individual markers as it is described in many breast cancer CTC studies.

4.3.2 Sensitivity and Specificity of Multimarker Assays

In order to verify if better sensitivity and specificity performances could be achieved in multimarker assays the combined expression of the four markers with the best sensitivity/specificity, namely CLDN7, CRYAB, ATP8B1 and EGFR was analyzed. Four multimarker (MM) patterns were established. Positivity of a blood sample was defined as one positive out of four tested genes in MM1, two positive out of four tested genes in MM2, three positive out of four tested genes in MM 3 and four positive out of four tested genes in MM4. From all MM patterns, MM1 showed the highest sensitivity (77.5%) and therefore was superior to the sensitivity of individual markers. However, the specificity in this MM pattern (80.0 %) was lower than the one from the best individual marker. All other multimarkers revealed lower sensitivities than single markers.

4.3.3 MM1 versus CRYAB

As stated before CRYAB was the most promising marker revealing the best relation of sensitivity and specificity of 35 % and 100 % respectively of all individual markers tested in this study. MM1 was the best multimarker pattern with the best sensitivity/specificity relation of 77.5 % and 80 % respectively. Hence the MM approach increased the sensitivity but at the cost of a decrease on the specificity. However, at this moment it was important to place the question of which of the two approaches is the most advantageous. Furthermore, it is also essential to reflect which feature is more relevant, the ability of the assay to detect the actual true positive results, i.e. the sensitivity, or the ability to identify the true negative results, i.e. specificity? The best option seems to consider the best relation of sensitivity and specificity and not any of these features singly. MM1 therefore appears to be the best option. Additionally, since CRYAB is already included in MM1, the inclusion of the detection abilities of three additional markers such as in MM1 can only be advantageous, since CMT may reveal varying expression profiles.

4.3.4 False-positive Results

The surprising observation of positive results for several markers in the PB blood of dogs with malignant MT without evidence of vascular invasion of tumor cells or benign neoplasias (group 2 and group 3) raises questions as to the origin of positivity. The number of positive results was particularly high for ELF3 in both group 2 and 3 (47.5 % and 42.5% respectively) and it is also questionable if these results are actually true false-positive results. First, it is important to consider possible contaminations with epidermal cells during blood sampling or the shed of tumor cells during palpation or manipulation of the tumors may be the source of the positive results in group 2 and 3 and it has been described before (Jiang et al., 2002b). Furthermore, it has been described that malignant tumors may actively shed tumor cells into circulation but because metastasis is an inefficient process that depends on several factors, these cells are not always able to successfully develop distant or regional metastases (Chambers et al., 2002; Sleeman et al., 2011). It is also possible that the vascular invasion of tumor cells is missed by the routine histological diagnosis and therefore that this positivity is not an actual false-positive result but just the detection of CTC that have escaped histological identification.

4.3.5 False-negative Results

Another questionable finding was the negative results in group 1. These potentially false-negative results may be due to two reasons.

First, CTC shed from the primary tumor have an average half-live of 1 to 2.4 hours and are considered rare events, occurring in numbers as low as 1 CTC per 10^7 to 10^5 PBL in human malignancies (Meng et al., 2004). Such estimations are still unavailable for dogs the dog but assuming that the number of CTC in dogs is similar, the assays used in this study should be sensitive enough to detect CTC. Nevertheless, at least in some dogs the CTC concentrations may be lower than in human patients and the assays therefore not sensitive enough. The introduction of an enrichment step prior to the detection step with RT-PCR could be a way of increasing sensitivity (Mostert et al., 2009). This enrichment step could include techniques such as density gradients in which tumor cells are separated from the PBL due to their physical properties or immunomagnetic enrichment in which tumor cells are separated from PBL through positive selection upon the expression of cell surface antigens such as EpCAM or negative selection with panleukocytic markers such as CD45 (Mostert et al., 2009). Nevertheless, canine specific enrichment assays are not available and the cross-species specificity of human assays for canine EpCAM has not been tested so far.

Second, CTC markers used in this study were identified using two MT cell lines (CMM26 and CMM115). It is probable that this two cell lines may not actually reflect the full range of possible gene expression patterns of mammary carcinoma and CTC in the dog, so that further studies might be necessary.

4.4 Conclusion and Outlook

From all markers tested CRYAB seems to be a highly specific but moderately sensitive marker for CTC detection in dogs with mammary carcinomas. Combination of multiple individual markers in multimarker assays such as MM1 (CLDN7, CRYAB, ATP8B1 and EGFR) allows an increased sensitivity but at the cost of a lower specificity to detect CTC in canine PB. Immunomagnetic CTC enrichment may be one option to overcome the sensitivity of the RT-PCR assays. Furthermore, the assays ability to differentiate blood from dogs with malignant tumors with and without vascular invasion with tumor cells shows the potential of these markers to dogs with CTC present in the PB and thus with a high risk to develop distant metastases.

This study was the first study focusing on CTC and their detection in the dog, however to fully determine the prognostic relevance of CTC in canine patients with MT, further standardized clinical studies using large cohorts of canine patients in order to achieve statistical significance necessary. This follow-up period should be of 24 month and should include periodical extensive physical examinations and imaging procedures (Matos et al., 2012). Only then disease-free and overall survivals can be determined and the relationship with the expression of CMT CTC markers can be established. Hopefully in the future CTC detection may be used for the prognosis of CMT or predict metastatic spread in these patients hence providing clinicians valuable information, that can help clinicians orientate therapeutical and palliative treatments.

Summary

Circulating Tumor Cells as Indicators of Metastatic Spread of Canine Mammary Tumors

Afonso da Costa

In breast cancer CTC have been subject of much attention in the past years. CTC and their detection have proven clinical usefulness in many types of cancers. In fact through CTC enumeration lower CTC counts were correlated with better disease-free and overall survivals in patients with breast cancer, before and after treatment, hence providing valuable information in terms of prognosis and response to treatment (Cristofanilli, 2006; Hayes et al., 2006; Mostert et al., 2009).

Based on in these findings it was hypothesized that CTC are also present in dogs baring metastatic mammary tumors (MT), their detection using nucleic-acid based methods is possible and may correlate with the histological evidence of vascular invasion by tumor cells in the vicinity of the primary tumor.

The first phase of the project consisted in the identification of potential canine specific MT CTC mRNA markers. To this end a total of 106 genes identified by three different approaches were tested for their sensitivity and specificity: (1). mRNA markers with proved usefulness for CTC detection of breast cancer CTC; (2). genes known to be overexpressed in CMT; (3). genes identified by microarray technology. These genes were then tested for their expression in the peripheral blood (PB) samples of healthy dog donors (n=12 to 14), metastatic mammary gland carcinomas (n=10) and malignant CMT cell lines (n=2) by RT-PCR. To be considered a potential CTC marker, the markers had to follow the selection criterion of not being expressed in the PB and to be expressed in metastatic carcinomas and malignant cells lines. A total of 12 potential candidate mRNA markers were identified. Furthermore the ability of these candidate mRNA markers to detect single tumor cells was tested in serial dilutions of tumor cells from cell lines CMM 26 and CMM115 in the PB were executed. Based on the frequency of occurrence of CTC in humans (1 CTC per 10^5 to 10^7 peripheral blood leukocytes (PBL)) a target sensitivity of 1 tumor cell per 10^6 to 10^7 PBL was established. From all the candidates only maspin was not able to achieve the desired sensitivity. In a second phase a panel of seven of the previously identified markers were tested in 120 PB samples from female dogs with malignant mammary gland carcinomas with histological evidence of vascular invasion of tumors cells near the primary tumor or presence of metastatic cells in the regional lymph node (n=40), with malignant mammary gland carcinomas without histological evidence of vascular invasion and metastasis free lymph

nodes (n=40) and patients with benign mammary gland adenomas (n=40), in order to correlate their expression with the existence of histological evidence of vascular invasion and therefore with the presence of tumor cells in vascular compartments. From the panel of seven markers tested CRYAB was the most promising marker revealing the best relation sensitivity/specificity. Through the establishment of multimarker assays combining the detection abilities of individual markers (CLDN7, CRYAB, ATP8B1 and EGFR) we were able to significantly increase the sensitivity. From all the multimarker patterns MM1 was the best displayed with the best sensitivity/specificity relation. Although multimarker assays significantly enhance the sensitivity of the assay, a moderate loss of specificity is observed, however the multimarker approach seems to be more advantageous the single marker approach. The assays ability to differentiate blood from dogs with malignant tumors with and without vascular invasion with tumor cells, suggest that they may allow the identification of dogs with CTC in the PB, nevertheless further studies that include a follow-up period of 24 month with regular physical examinations and imaging scans are required to determine the real prognostic significance of these CTC markers in the dog.

Zusammenfassung

Zirkulierenden Tumorzellen als Indikatoren der Metastasierung der caninen Mammatumoren

Afonso da Costa

Zirkulierende Tumorzellen (CTC) waren in den letzten Jahre vermehrt Gegenstand wissenschaftlicher Untersuchungen in der Brustkrebsforschung. So konnte bei Brustkrebspatientinnen gezeigt werden, dass niedrige CTC-Zahlen im Blut mit einem längerem Überleben korrelieren (Cristofanilli, 2006; Hayes et al., 2006; Mostert et al., 2009).
Basierend auf diesen Erkenntnissen wurden für die vorliegende Arbeit folgende Hypothesenaufgestellt: (1). CTC treten auch bei Hunden mit metastasierenden Mammatumoren (MT) auf, (2). ihr Nachweis ist mit Nukleinsäure-basierten Verfahren möglich und (3). der Nachweis vonCTC korreliert mit dem histologischen Nachweis von Gefäßeinbrüchen von Tumorzellen inder Nähe des Primärtumors korreliert ist.
Die erste Phase des Projekts umfasst die Identifizierung potenzieller, hundespezifischer MT CTC mRNA-Marker. Zu diesem Zweck wurden insgesamt zahlreiche Gene getestet, die durch drei Ansätze identifiziert wurden: (1) mRNA-Marker mit nachgewiesener Eignung zur Erkennung von Brustkrebs CTC; (2) Gene mit bekannter Überexpression in CMT und (3) Gene, die durch Microarray-Technologie identifiziert wurden. Diese Gene wurden dann durch RT-PCR auf ihre Expression im peripheren Blut (PB) gesunder Hunde (n=12 bis 14), Hunden mit metastasierenden Mammakarzinomen (n=10) und malignen CMT-Zelllinien (n=2) getestet. Um als potenzieller CTC Marker zu gelten, sollten die Marker nicht im PB, jedoch immer in metastatischen Karzinomen und MT-Zelllinien exprimiert werden. Insgesamt wurden 12 potentielle mRNA-Marker selektiert. Diese wurden anschließend auf ihre Sensitivität zum Nachweis von CTC in Verdünnungsreihen von Tumorzellen aus zwei Zelllinien im PB getestet. Dabei wurde eine Empfindlichkeit von 1 Tumorzelle pro 10^6 bis 10^7 Leukozyten angestrebt. Von allen Kandidaten konnte nur Maspin nicht die gewünschte Sensitivität erreichen.
In einer zweiten Phase der Untersuchungen wurden sieben der identifizierten Marker in 120 Blutproben von Hündinnen mit Mammakarzinomen mit histologischem Nachweis metastasierenden Verhaltens (n=40), Hündinnen mit Mammakarzinomen ohne histologischen Nachweis metastasierenden Verhaltens (n=40) und Hündinnen mit Adenomen der Milchdrüse (n=40) getestet Dabei zeigte sich CRYAB als der sensitivste Marker für kanine CTC. Weiterhin zeigte CRYAB das beste Verhältnis von Sensitivität zu Spezifität des Nachweises von CTC. Eine verbesserte Kombination aus Sensitivität und Spezifität konnte

jedoch durch die Etablierung eines Multimarker aus 4 Genen, CLDN7, CRYAB, ATP8B1 und EGFR erreicht werden. Nichtsdestotrotz führte die höhere Sensitivität des Multimarkers zu einem moderaten Verlust an Spezifität im Vergleich zum Einzelmarker CRYAB.
Die statistischen signifikanten Unterschiede in der Häufigkeit der Expression der Marker in Blut von Hunden mit und ohne malignen Mammatumoren zeigen, dass CTC auch beim Hund wichtige Information zu Therapieerfolgen und Prognose von kaninen Mammatumoren beitragen können. Dennoch sind in Zukunft weitere prospektive klinische Studien nötig, um die prognostische Signifikanz dieser CTC Marker beim Hund sicherzustellen.

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Oral Presentations

"Circulating Tumor Cells in Dogs with Mammary Gland Tumors"

A. da Costa, B. Kohn, A.D. Gruber, R. Klopfleisch

Dahlem Research School Oral Presentation Seminar, Freie Universität Berlin, Berlin, Germany (07.07.2009)

"mRNA Markers for the Detection of Circulating Tumor Cells in Dogs with Metastatic Gland Tumors"

A. da Costa, B. Kohn, A.D. Gruber, R. Klopfleisch

2. PhD Presentation Seminar of the Dahlem Research School Graduate Studies Biomedical Sciences & Molecular Science at the Freie Universität Berlin, Berlin, Germany (25.05.2010)

"Genetic Markers for the Detection of Circulating Tumor Cells in Dogs with Metastatic Mammary Tumors"

A. da Costa, J. T. Oliveira, F. Gärtner B. Kohn, A.D. Gruber, R. Klopfleisch

28. Annual Meeting of the European Society of Veterinary Pathology and European Conference of Veterinarian Pathologists, Belgrade, Serbia (08.-11.09.2010)

"mRNA Markers for the Detection of circulating Tumor Cells in Dogs with Metastatic Gland Tumors"

A. da Costa, B. Kohn, A.D. Gruber, R. Klopfleisch

54. Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (12.-13.03.2011)

"mRNA Markers for the Detection of circulating Tumor Cells in Dogs with Metastatic Gland Tumors"

A. da Costa, B. Kohn, A.D. Gruber, R. Klopfleisch

55. Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (09.-11.03.2012)

Publications List

da Costa, A., Oliveira, J.T., Gärtner, F., Gruber, A.D., , Klopfleisch, R., 2011. Potential markers for detection of circulating canine mammary tumor cells in the peripheral blood. The Veterinary Journal, 190:165-168

da Costa A., Lenze, D., Hummel, M., Kohn, B., Gruber, A.D., Klopfleisch, R., 2012. Identification of Six Potential Markers for the Detection of Circulating Canine Mammary Tumor Cells in the Peripheral Blood Identified by Microarray Analysis. Journal of Comparative Pathology, Feb-Apr;146(2-3):143-51

da Costa A., Kohn, B., Gruber, A.D., Klopfleisch, R., 2012. Multiple RT-PCR Markers for the Detection of Circulating Tumour Cells of Metastatic Canine Mammary Tumours. The Veterinary Journal, Apr;196(1):34-9.

Acknowledgements

I would like to thank my mentor's team Prof. Dr. Robert Klopfleisch, Prof. Dr. Gruber and Prof. Dr. Barbara Kohn for allowing me to engage in this extraordinary journey that allowed me grow not only as a young scientist but also personally. I would like to thank them for their scientific and personal advice from which I have learned a lot.

I would like to thank Monica Schaerig for her technical support and her endless patience.

I would like to thank the Portuguese Foundation of Science and technology for the generous financial support.

I would also like to thank my very dear friend Dr. Sophie Bader for being there in the good and bad times.

Finally I would like to thank my parents for always being there for me.

Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 18.10.2012 Afonso da Costa