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Marie-Charlotte von Deetzen, geborene Frielitz
Tierärztin aus Berlin

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Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Robert Klopfleisch
Zweiter Gutachter:	PD Dr. Kerstin Müller
Dritter Gutachter:	PD Dr. Christine E. Hellweg

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List of abbreviations

A	**a**denosine
bp	**b**ase **p**airs
BRET	**b**ioluminescence **r**esonance **e**nergy **t**ransfer
C	**c**ytidine
CMT	**c**anine **m**ammary **t**umor/s
DFS	**d**isease-**f**ree **s**urvival
DGCR8	Pasha/**D**i**G**eorge syndrome **c**ritical **r**egion gene **8**
DNA	**d**esoxyribo**n**ucleic **a**cid
dT	**t**hymidine
ERBB3	**er**ythro**b**lastic leukemia viral oncogene **3**
G	**g**uanosine
GAS5	**g**rowth **a**rrest-**s**pecific transcript **5**
HuR	**hu**man antigen **R**
LNA	**l**ocked **n**ucleic **a**cid
miRNA	**mi**cro **r**ibo**n**ucleic **a**cid
mRNA	**m**essenger **r**ibo**n**ucleic **a**cid
MVD	**m**icro**v**essel **d**ensity
nt	**n**ucleo**t**ides
OS	**o**verall **s**urvival
PCR	**p**olymerase **c**hain **r**eaction
pre-miRNA	**pre**cursor **mi**cro **r**ibo**n**ucleic **a**cid
pri-miRNA	**pri**mary **mi**cro **r**ibo**n**ucleic **a**cid
PTEN	**p**hosphatase and **ten**sin homolog
RISC	**ri**bo**n**ucleic **a**cid **i**nduced **s**ilencing **c**omplex
RNA	**r**ibo**n**ucleic **a**cid
rRNA	**r**ibosomal **r**ibo**n**ucleid **a**cid
RT	**r**everse **t**ranscription
RT-PCR	**r**everse **t**ranscriptase **p**olymerase **c**hain **r**eaction
snoRNA	**s**mall **n**ucle**o**lar **r**ibo**n**ucleic **a**cid
snRNA	**s**mall **n**uclear **r**ibo**n**ucleic **a**cid
TARBP2	**t**rans**a**ctivation **r**esponse ribonucleic acid **b**inding **p**rotein **2**
TNM	**t**umor size, lymph **n**ode status, distant **m**etastasis
tRNA	**t**ransfer **r**ibo**n**ucleid **a**cid

1 INTRODUCTION

1.1 Morphology of the canine mammary gland

The mammary gland of the dog is composed of usually five mammary complexes bilaterally parallel to the abdominal midline. A mammary complex is defined as the physically circumscribable gland tissue which ends in one teat. Beginning with the most cranial one, the mammary complexes are called cranial and caudal thoracic complex, cranial and caudal abdominal complex and inguinal complex (Miller, Christensen et al. 1964; Barone 1990). Evolutionary the mammary gland is a modified sweat gland with a tubuloalveolar formation. Single lobules are supported by interlobular connective tissue. The surrounding stroma also contains nerves, vessels and in the juvenile gland abundant adipose tissue (Silver 1966; Salomon 2008).

The alveoli represent the parenchyma. The intralobular ducts arise from the alveoli and open into the interlobular lactiferous ducts, which in turn lead into the lactiferous sinus. The lactiferous sinus passes into the teat sinus, which opens onto the teat surface. In the dog, altogether eight to twelve papillary ducts per teat open onto the teat surface.

Depending on the secretory activity the alveolar epithelium is simple cuboidal to columnar. Between the alveolar epithelium and its basal lamina the characteristic star-shaped myoepithelial cells are present. The myoepithelial cells are contractile and therefore fascilitate the milk ejection. The ductal epithelium differs according to the duct size. The intralobular and small interlobular ducts are secretory and lined by a simple cuboidal epithelium. Larger ducts and the sinuses otherwise are lined by bistratified cuboidal to columnar epithelium. The papillary ducts have keratinized stratified squamous epithelium, which continues into the skin surface of the teat (Silver 1966; Barone 1990; Salomon 2008).

The blood supply of the mammary gland is made up of different arteries, which also anastomose with each other. The thoracic mammary complexes are supplied by the cranial superficial epigastric artery and additionally by branches of the lateral thoracic and intercostal arteries. The cranial abdominal mammary complexes receive blood by the cranial superficial epigastric artery and anastomoses of the caudal superficial epigastric artery. The caudal superficial epigastric artery also supplies the caudal abdominal and the inguinal complexes (Silver 1966; Salomon 2008). Both caudal mammary glands also receive blood via small branches of different other arteries (Silver 1966). Besides the craniocaudal anastomoses of the cranial and the caudal superficial epigastric artery, laterolateral anastomoses of few

arteries are described (Silver 1966). The venous outflow of the mammary gland is similar to the arterial supply. However, the more voluminous veins show more craniocaudal and laterolateral anastomoses than the arteries (Silver 1966). The cranial and caudal thoracic mammary glands drain into the cranial superficial epigastric vein as well as branches of internal thoracic veins and intercostal veins. The venous blood of both abdominal and the inguinal mammary glands is drained by the caudal superficial epigastric vein (Silver 1966; Salomon 2008).

The literature concerning the lymphatic drainage of the mammary gland is contradictory. Of which the relationship of mammary complexes to lymph centres and the existence of anastomoses is inconsistently described. Generally, each mammary complex is drained via many small lymphatics, which continue together with a similar sub-dermal lymphatic network into larger lymph vessels which finally lead to the draining lymph node (Silver 1966). Several draining lymph nodes can be summed up to a lymph centre. Thus, both thoracic mammary complexes commonly drain to the ipsilateral axillary lymph centre, which consists of the proper axillary lymph node and the accessory axillary lymph node if present. The caudal abdominal and the inguinal mammary complexes usually drain to the ipsilateral inguinofemoral lymph centre, which consists of the superficial inguinal lymph nodes, also called mammary lymph nodes. The cranial abdominal mammary complexes drain both to the axillary and the inguinofemoral lymph centres (Patsikas and Dessiris 1996a; Patsikas and Dessiris 1996b; Pereira, Rahal et al. 2003; Patsikas, Karayannopoulou et al. 2006). Many studies reveal different variations of the lymphatic drainage pattern. Similar to the arteries and veins, the lymph vessels can anastomose ipsilaterally and contralaterally (Pereira, Rahal et al. 2003). However, Patsikas and colleagues even showed a retrograde lymph flow through the regional lymph node from one mammary gland to another and besides that a contralateral connection between both superficial inguinal lymph nodes (Patsikas and Dessiris 1996a; Patsikas and Dessiris 1996b). Figure 1 illustrates both the general lymphatic drainage pattern of the canine mammary gland and variations in the lymph flow described by Patsikas et al..

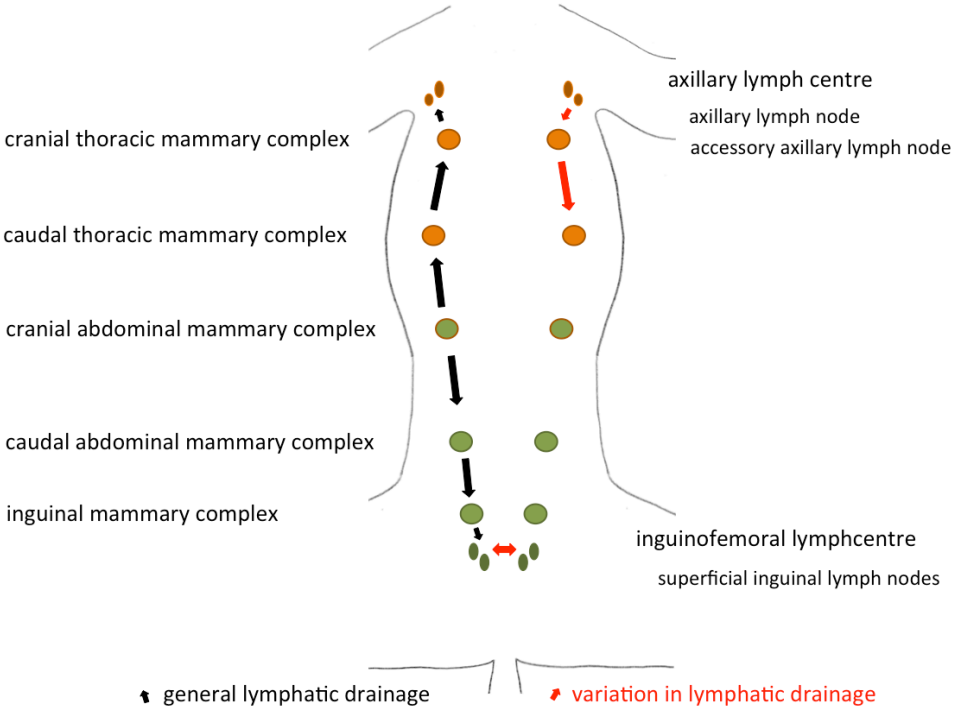

Figure 1: Lymphatic drainage of the mammary gland

An interesting finding is that the drainage pattern can be altered in neoplastic mammary glands by forming more anastomoses when compared to non-neoplastic glands (Pereira, Rahal et al. 2003; Patsikas, Karayannopoulou et al. 2006). Therefore, besides the drainage to the classic lymph centres retrograde and contralaterally metastatic spread via lymphatics is possible. This finding should be taken into account by the clinician in determining the lymph nodes, which have to be evaluated for neoplastic spread. However, lymph nodes of the classic ipsilateral lymph centres are still the focus in mammary tumor diagnosis and the functional relevance of new drainage channels formed by the mammary tumor remains questionable.

1.2 Prevalence and occurrence of canine mammary tumors

Canine mammary tumors (CMT) are the second most common neoplasms in dogs next to skin tumors (Moulton 1990) and even the most common neoplasms in the female dog since CMT are representing more than 40 % of all tumors in the bitch (Dorn, Taylor et al. 1968). The incidence of CMT is regionally variable. In regions where it is an established practice to spay female dogs in the early years of age, the incidence of CMT is decreasing whereas it is increasing in regions where preventive neutering is uncommon (Moe 2001; Egenvall, Bonnett et al. 2005). The most affected mammary complexes are the cranial abdominal and inguinal

ones. Fifty to approximately 70 % of the affected dogs show multiple CMT (Benjamin, Lee et al. 1999; Misdorp 2002; Sorenmo 2003; Sorenmo, Kristiansen et al. 2009). These multiple CMT are often of variable size and histopathology (Sorenmo, Rasotto et al. 2011). About 50 % of the mammary tumors are histologically diagnosed as malignant (Hampe and Misdorp 1974; MacEwen 1996). Lymphogenous and haematogenous metastases are common and metastatic disease is the most frequent cause of death in these patients (Misdorp 1976; Moulton 1990).

1.3 Risk factors

Several factors may influence the risk of developing CMT. The most important risk factors are age, breed, sex, hormones, diet, and pre-malignant lesions.

The age distribution of CMT is very similar to the distribution of most canine tumors (Moulton 1990). They are rare in dogs under two years of age, whereas the incidence is increasing at the age of six years, and decreases again in dogs older than ten years. The onset of CMT correlates well with the onset of human breast cancer regarding the age conversion table of Lebeau (Lebeau 1953) with a correction for giant and very small breeds (Moulton 1990). Mostly affected are middle-aged to old dogs with a median age of occurrence ranging from eight to ten years (Schneider 1970; Hellmen, Bergstrom et al. 1993; Chang, Chang et al. 2005). Interestingly, the tumor onset is different between benign and malignant CMT (Moulton 1990). For the occurrence of benign CMT a mean age of 8.5 years is described, whereas the mean age of occurrence for malignant CMT is 9.5 years (Sorenmo, Kristiansen et al. 2009).

The influence of breed is ambiguous. There are partially contradicting descriptions of breed predisposition (MacEwen 1996; Borge, Borresen-Dale et al. 2011). However, these associations might be biased by regional breed popularity and the fact, that the age conversion between giant and small breeds versus middle sized breeds is not proportional (Moulton 1990). CMT appear earlier in life in giant and small breeds than in middle sized dogs. Thus, the assumed breed predisposition might rather be a reflection of the age of tumor onset (Moulton 1990; Schafer, Kelly et al. 1998).

The development and growth of the healthy mammary gland is controlled by endogenous ovarian steroid-hormones, both oestrogen and progesterone. The proliferative effect of these hormones may also promote neoplastic growth (Thomas 1984; Queiroga, Perez-Alenza et al. 2005). While oestrogen stimulates ductal proliferation, progestin is reported to promote

lobuloalveolar development and hyperplasia of both secretory and myoepithelial cells (Rutteman 1990). It is hypothesized that the tumor-promoting effect of progesterone is associated with growth hormone up-regulation, which may stimulate the proliferation of mammary stem cells (Mol, van Garderen et al. 1996; van Garderen, van der Poel et al. 1999).

The effect of both steroid hormones, oestrogen and progesterone, is mediated by receptors. Oestrogen and progesterone receptors are expressed both in healthy and neoplastic mammary tissues (MacEwen, Patnaik et al. 1982; Rutteman, Misdorp et al. 1988; Chang, Tsai et al. 2009). Both receptors are decreased in malignant CMT when compared to benign ones (MacEwen, Patnaik et al. 1982; Rutteman, Misdorp et al. 1988; Chang, Tsai et al. 2009). The serum levels of the steroid hormones are inversely correlated with the presence of steroid receptors. Thus the serum levels of bitches with malignant CMT are significant higher than in bitches with benign CMT or normal mammary glands (Queiroga, Perez-Alenza et al. 2005). High levels of both oestrogen and progestin, e.g. by exogenous administration of hormone derivates, are described to increase the mammary tumor risk (Giles, Kwapien et al. 1978). In turn, diminished levels of ovarian steroid hormones as induced by spaying are proposed to decrease this risk.

One of the most citied publication on the effect of ovariohysterectomy by Schneider et al (Schneider, Dorn et al. 1969) illustrated that a bitch, which is spayed prior the first oestrus, has a 0.5 % risk of developing mammary tumors compared with sexually intact ones. Bitches that are spayed between the first and second oestrus have a risk of 8 %. Ovariohysterectomy after the second oestrus leads to a 26 % risk in bitches that are younger than two and a half years. This study stated that later spaying does not influence the risk for developing CMT, whereas other studies even showed a protective effect of later spaying (Taylor, Shabestari et al. 1976; Sonnenschein, Glickman et al. 1991). However, a recent study conducted a systematic review on the effect of spaying and concluded that both the effect of neutering itself and the age at neutering on the risk of developing mammary tumors has to be graded only weak. The authors criticized that most literature on this topic is several decades old and neither current epidemiological methods nor the impact of potential biases had been considered adequately (Beauvais, Cardwell et al. 2012). Other reproductive circumstances like oestrus irregularity, pseudo-pregnancy, pregnancy and parity seem to have no effect on mammary tumor risk in dogs (Brodey, Fidler et al. 1966; Schneider, Dorn et al. 1969). This is in contrast to women, where a late age of first birth and low parity is assumed to be associated with an enhanced risk for developing breast cancer (Kvale 1992).

The role of body condition and diet as a risk factor for mammary cancer is evaluated in several human and veterinary studies. Dogs that were thin at the age between nine and twelve months, have a decreased mammary tumor risk (Sonnenschein, Glickman et al. 1991). However, obesity one year prior to tumor diagnosis or high-fat diet had no influence on the mammary tumor risk in this study. In contrast, another retrospective study could show that obesity at one year of age and high red meat intake represent independent risk factors for developing CMT and mammary gland dysplasia (Alenza, Rutteman et al. 1998). Nevertheless, obesity, same as the influence of steroid hormones, seem to have the strongest effect on developing CMT in the early lifetime of dogs (Sorenmo, Rasotto et al. 2011). In postmenopausal women the correlation between obesity and developing breast cancer is the same. The effect of obesity may be based on both increased circulating free oestrogen and increased local oestrogen production via the enzyme aromatase (Stephenson and Rose 2003; Cleary and Grossmann 2009; Cleary, Grossmann et al. 2010). This underlines the effect of hormones on tumor development.

Premalignant lesions like atypical hyperplasia or carcinoma-*in-situ* in women and bitches are correlated with an increased risk for developing new primary mammary tumors (Arpino, Laucirica et al. 2005; Hartmann, Sellers et al. 2005; Stratmann, Failing et al. 2008; Sorenmo, Kristiansen et al. 2009; Mouser, Miller et al. 2010). These new mammary tumors can occur on the ipsilateral side and less often on the contralateral side. These findings lead to the assumption, that premalignant lesions represent precursors of breast cancer and CMT, respectively (Allred, Mohsin et al. 2001; Hartmann, Sellers et al. 2005; Stratmann, Failing et al. 2008; Sorenmo, Kristiansen et al. 2009; Mouser, Miller et al. 2010).

1.4 Diagnosis of CMT

The clinical examination of dogs presented with one or more nodules within the mammary gland should include a complete clinical history, physical examination of the dog with careful palpation of all mammary glands and the draining lymph nodes (MacEwen 1996; Misdorp 2002; Sorenmo, Rasotto et al. 2011). CMT occur multiple in almost 70 % of the patients, and the most commonly affected mammary complexes are the caudal ones (Sorenmo, Kristiansen et al. 2009). Synchronous tumors often show a variable size and histopathology. Thus, every single nodule should be clinically and histologically examined. Depending on the biological behavior and stage of the CMT the nodules can occur in variable size, free movable or fixed, ulcerated, and single or multiple (Sorenmo, Rasotto et al. 2011).

Generally, the patients show no signs of systemic illness, with the exception of advanced metastatic disease or the uncommon but extremely aggressive entity of inflammatory mammary carcinoma. In these dogs blood chemistry may be altered, including coagulopathies. Signs of systemic illness are non-specific and include lethargy, anorexia, weight loss, dyspnoea, cough, lymphedema or lameness (MacEwen 1996; Misdorp 2002; Marconato, Romanelli et al. 2009). Metastatic dissemination happens most commonly via the lymphatics to the draining lymph node and the lung, though haematogenous spread of metastatic tumor cells is also evident. Liver, bone and brain are further but less common sites of metastases (MacEwen 1996; Chambers, Groom et al. 2002; Misdorp 2002). Careful palpation of the draining lymph node is obligatory to ascertain enlargement. Fine needle aspiration with subsequent cytological examination or better complete excision of the lymph node together with the affected mammary glands and subsequent histopathological examination should be conducted as a part of tumor staging (Tuohy, Milgram et al. 2009). Additional three-way thoracic radiographs and abdominal ultrasound are recommended to exclude lung or abdominal metastases (Otoni, Rahal et al. 2010). Cytological examination of CMT is useful to differentiate CMT from other causes of mammary gland swelling and to distinguish CMT from other tumors. However, cytology may be impaired by a relative high proportion of non-tumor cells due to extensive stromal reaction or infiltration of inflammatory cells. Thus, histopathological examination is considered to be the gold standard for diagnosis and classification of mammary gland tumors (Sorenmo, Rasotto et al. 2011).

1.5 Prognosis of CMT

CMT is the most common tumor in the female dog, and up to 50 % of the mammary tumors are malignant (Hampe and Misdorp 1974; MacEwen 1996). To predict the clinical outcome in the years after surgery, many studies have been conducted to reveal prognostic factors for CMT. The setup of a valid classification system for a reliable prognosis is hindered by the heterogeneous biological behavior of CMT. Several pathological features of CMT are identified as consistent prognostic factors including clinical stage, tumor size, lymph node status, presence of distant metastasis, histological grade, and microvessel density. Besides these gross and histopathological features, the predictive potential of various molecular markers such as oncogenes, tumor suppressors, adhesion molecules and proliferation markers have been investigated (Klopfleisch, von Euler et al. 2011).

1.5.1 Clinical staging

Clinical staging is a tool to find the adequate therapy for the specific patient in regard to the prognosis of the CMT. Two classification systems for CMT have been published. The first one is the original World Health Organization (WHO) staging system (Owen 1980) and the second one represents a modified WHO staging system (Rutteman, Withrow et al. 2001). In both classification systems CMT are staged regarding to **t**umor size, lymph **n**ode status and presence of distant **m**etastasis (TNM system). Therefore, it is mandatory to inspect every single tumor nodule and the draining lymph node as well as to search for distant metastasis as described above. In the following, only the more recent modified WHO staging system will be explained. This TNM system implies five stages with increasing malignancy from stage I to stage V. Stage I, II and III represent CMT of different size with neither lymph node involvement nor distant metastasis. Stage IV is characterized by the presence of lymph node metastasis independent of the primary tumor size and without distant metastasis. Stage V is defined by distant metastasis regardless the tumor size or lymph node status (Rutteman, Withrow et al. 2001).

Modified WHO staging system					
Status	I	II	III	IV	V
Primary tumor size	< 3 cm	3 – 5 cm	> 5 cm	any size	any size
Lymph node status	no	no	no	yes	no / yes
Distant metastasis	no	no	no	no	yes

Table 1: Modified WHO staging system (TNM system)

The prognostic value of the TNM staging system, measured as post-operative survival time (OS), is supported by several studies. Chang and colleagues could show a significant difference in survival time between the stages I, II and III on the one hand and stage IV and V on the other hand (Chang, Chang et al. 2005). In another study, dogs with clinical stage I had the longest median survival time when compared with stages II, III and IV (Philibert, Snyder et al. 2003).

1.5.2 Tumor size

Tumor size is one of the determinants of the clinical staging system. Furthermore, the prognostic significance of tumor size alone is generally assumed. The disease-free survival time (DFS), defined as the time from surgery to occurrence of metastasis or recurrence, and the post-operative survival time (OS) are reported to be shorter in dogs with larger CMT.

More precisely, dogs with CMT larger than 3 cm in diameter have significantly decreased DFS and OS than dogs with CMT smaller than 3 cm in diameter (Kurzman and Gilbertson 1986; Perez Alenza, Pena et al. 1997; Philibert, Snyder et al. 2003). An increasing tumor size may also be associated with other factors indicating a poor prognosis. Thus, a tumor size larger than 3 cm in diameter is often correlated with an increased proliferation index or decreased expression of progesterone receptors (Ferreira, Bertagnolli et al. 2009; Sorenmo, Kristiansen et al. 2009). Furthermore, the likelihood of lymph node metastasis is increased in CMT larger than 5 cm in diameter (Chang, Chang et al. 2005). The size limits in these studies match the size categories of the modified TNM staging system.

1.5.3 Lymph node status and distant metastasis

Metastasis is the most frequent cancer-related cause of death in both women and dogs (Misdorp 1976; Kim, Oh et al. 2011; Ru, Steele et al. 2011). Metastasis to the regional lymph node and distant metastasis also confer prognostic relevance. The evidence of metastasis at the time of diagnosis is related to a poorer prognosis (Yamagami, Kobayashi et al. 1996; Philibert, Snyder et al. 2003; Karayannopoulou, Kaldrymidou et al. 2005). In a two-year clinical follow-up study, 85.7 % of the dogs with manifest lymph node metastasis died in contrast to 21.1 % of the patients without nodal metastasis (Karayannopoulou, Kaldrymidou et al. 2005). Another study correlated the influence of distant metastasis with the survival time. It showed that dogs with metastasis had a median post-operative survival time of 5 months to a survival time of 28 months of dogs without metastasis (Philibert, Snyder et al. 2003).

The metastatic cascade is a very complex process in which the tumor cells have to complete several steps. This includes the entrance and survival in the circulatory system, settling and extravasation at a new site, initiation and maintenance of growth (Chambers, Groom et al. 2002; Jiang, Martin et al. 2002). Within the primary tumor, which is characterized by unregulated growth and exhibits various genetic abnormalities, a subset of tumor cells is assumed to show metastatic properties like motility and invasiveness during tumor development (Bednarz-Knoll, Alix-Panabieres et al. 2011). However, it remains unclear, if the ability for forming metastasis is a feature, which the tumor acquires in the course of malignant progression or if the capacity for metastatic spread is inherent in a particular tumor from the very beginning of tumor development even before metastatic spread actually occur (van 't Veer, Dai et al. 2002).

1.5.4 Histological type and grade

The classification of CMT according to the WHO (Misdorp, Else et al. 1999; Goldschmidt, Pena et al. 2011) is based on histological criteria. The most significant criteria for malignancy are nuclear and cellular pleomorphism, the mitotic index, presence of necrosis within the neoplasm, invasive tumor growth and regional lymph node metastasis (Misdorp 2002; Goldschmidt, Pena et al. 2011). By means of these criteria CMT can be classified into benign and malignant tumors, whereas the correlation between inflammatory cell infiltration and malignancy is still ambiguous (Misdorp 2002; Estrela-Lima, Araujo et al. 2010). Another aspect for CMT classification is the cell of origin, on this basis CMT can grossly be distinguished into epithelial tumors, mesenchymal tumors or tumors with both epithelial and mesenchymal differentiation, also called mixed tumors. Malignant mesenchymal tumors, also called sarcomas, comprise fibrosarcoma, osteosarcoma, carcinosarcoma, sarcoma in benign tumors and other sarcomas. Malignant epithelial tumors, also called carcinomas, include carcinoma in situ, complex carcinoma, simple carcinoma and carcinomas of special type like squamous cell carcinoma and mucinous carcinoma. Carcinoma in situ is defined as a well-demarcated malignant epithelial tumor with no extension through the basement membrane. Tumors of the complex type have components of both epithelial and myoepithelial cells. Simple carcinomas are further sub-divided into tubulopapillary, solid and anaplastic carcinoma (Misdorp, Else et al. 1999). A carcinosarcoma is a malignant mixed mammary tumor which is composed of cells resembling both epithelial cells and connective tissue (Misdorp, Else et al. 1999).

Most of the malignant CMT are carcinomas and less than 5 % are sarcomas (MacEwen 1996). Sarcomas are reported to be the most malignant tumor type with increasing malignancy from complex carcinoma over simple carcinoma to sarcoma. (Misdorp 1976; Else and Hannant 1979; Parodi, Misdorp et al. 1983; Hellmen, Bergstrom et al. 1993). Benign canine mammary gland tumors are grouped into adenoma, fibroadenoma, benign mixed tumors and duct papilloma (Misdorp, Else et al. 1999).

Although the classification of CMT follows a clear scheme, a reliable prognosis remains a challenge. This concerns particularly the early differentiation between non-metastasizing carcinoma and metastasizing carcinoma prior to histological evident metastasis. The limitations of this CMT classification is also underlined by the assumption that approximately 10 % of CMT are malignant tumors misdiagnosed as benign ones (Hampe and Misdorp 1974).

The Elston/Ellis grading system (Elston and Ellis 1991) is a tool to assess the level of breast cancer differentiation. Its three grading criteria are tubular formation, nuclear pleomorphism

and mitotic count. Each feature is given one to three points. Scores for each feature are added up and result in a final score, which corresponds to the grade. Grade I is a well-differentiated tumor with a final score of three to five points. Grade II is a moderately differentiated tumor with a final score of six or seven points. Grade III is a poorly differentiated tumor with a final score of eight or nine points.

Tumor grading schedules like the Elston/Ellis system are not generally accepted in the veterinary medicine. However, a two-year follow-up study by Karayannopoulou and colleagues could show that the post-operative survival time of dogs with CMT grade III was significant shorter than in patients with CMT grade II or grade I. A decreasing level of differentiation was correlated with an increasing risk of death. The authors recommended the routine use of the human Elston/Ellis grading system for accurate prognosis of CMT (Karayannopoulou, Kaldrymidou et al. 2005).

1.5.5 Microvessel density

The development of blood vessels from preexisting vessels, called angiogenesis, is a crucial process for tumor growth and metastatic dissemination (Folkman 1990). The tumor microvasculature is measured by the microvessel density (MVD). MVD is reported to be correlated with the likelihood of forming metastasis, tumor recurrence and survival. The prognostic impact of MVD could be shown for different human neoplasms including breast and prostate cancer (Weidner, Semple et al. 1991; Borre, Offersen et al. 1998; de Jong, van Diest et al. 2000). In CMT, a significant higher MVD count was observed in malignant tumors with metastatic spread when compared to benign tumors. Furthermore, a correlation of MVD with the histological tumor type, tumor grade and survival could also be shown for CMT (Graham and Myers 1999; Restucci, De Vico et al. 2000; Millanta, Silvestri et al. 2006).

1.5.6 Limitation of diagnosis and prognosis

Several tumor features have been investigated to establish an accurate diagnosis and reliable prognosis for CMT. The occurrence of metastasis as one of these features has a major impact on survival. Therefore, the early differentiation between non-metastasizing carcinoma and metastasizing carcinoma prior to evident metastatic spread plays a key role for a reliable prognosis of CMT. On this account, histopathological examination should include conscientious evaluation of cutting margins, lymphatics and blood vessels as well as the draining lymph node.

However, due to appreciable variations in the biological behavior of CMT - even within the same histological tumor type - the exclusive histopathological diagnosis is often insufficient for prognosis and therapy (Sarli, Preziosi et al. 2002). Van't Veer and colleagues showed that actually small, non-metastasizing primary breast tumors exhibit a gene expression signature which is associated with poor prognosis (van 't Veer, Dai et al. 2002). Therefore, the conclusion can be drawn that the metastatic potential of a particular CMT is already programmed before actual metastatic spread. This indicates the increasing need for diagnostic strategies besides the classic histopathological features that facilitate a prediction of metastatic potential of the tumor before metastases actually occur. In recent years, several diagnostic targets on the molecular level have been investigated to elucidate essential factors for neoplastic transformation, malignant progression and metastatic potential. The molecular carcinogenesis of CMT has been explored on the level of genes, RNA and proteins.

1.6 Molecular carcinogenesis

Tumor development is a multistep process, which includes three major stages, called initiation, promotion, and progression. During the first step, the initiation, a mutational event leads to an irreversibly altered cell. Promotion is the clonally expansion of the initiated cell. Further clonal selection and proliferation of the tumor cells is called progression. All of these stages are likewise essential for tumor evolution (Barrett 1993). In contrast to the non-neoplastic tissue, cancer is characterized by an uncontrolled cellular proliferation and furthermore depending on the stage of malignancy by the capability for tissue invasion and metastatic spread (Mocellin, Pasquali et al. 2009). In non-neoplastic tissue, cell proliferation is strictly regulated by several molecular pathways, e.g. the cell cycle checkpoints, apoptosis, and DNA repair systems (Sarli, Preziosi et al. 2002).

All the mentioned regulatory circuits are predicated on the expressional "switch on" and "switch off" of corresponding genes. However, deregulation of these genes is assumed to be accountable for the first step in tumor development (Croce 2008). According to the particular effect in carcinogenesis, the involved genes are grouped into oncogenes and tumor suppressor genes. More precisely, up-regulation of oncogenes like cyclin A and cyclin D1 or down-regulation of tumor suppressors like the phosphatase and tensin homolog (*PTEN*) can promote tumor growth (Murakami, Tateyama et al. 2000; Zhang, Piccini et al. 2012). The genetic instability of proliferating tumor cells seems to accumulate in the course of malignant progression. This in turn may cause further mutational activation of oncogenes and inactivation of tumor suppressors (Callahan and Campbell 1989). Thus, carcinogenesis

represents an evolutionary process of repeated rounds of mutation. This is supported by the cancer stem cell theory. Corresponding to the cancer stem cell theory a tumor is not a homogenous, solid mass, but instead consists of different, indefinitely replicating stem cells and their restrictively proliferating daughter cells (Gilbertson and Graham 2012). The tumor stem cells have the capability to propagate mutations and thus are drivers of carcinogenesis (Greaves and Maley 2012).

Until recently, tumor suppressor genes and oncogenes were thought to be restricted to protein coding genes. However, recent studies indicate that also regulatory RNA species are involved in tumor development and may function as oncogenes and tumor suppressors, respectively (Rossi, Sevignani et al. 2008). Molecules involved in the different steps of carcinogenesis can function as mediators of apoptosis and DNA repair, adhesion molecules, and mediators of angiogenesis (Klopfleisch, von Euler et al. 2011). These molecules can be investigated on the genomic, RNA and protein level. Certain coding genes and key proteins are found to be deregulated in cancer. Conspicuously, the messenger RNA (mRNA) expression often correlates poorly with the protein level. Therefore, the relevance of mRNA expression patterns has to be further investigated (Klopfleisch, von Euler et al. 2011). However, besides the protein coding RNA, the transcriptome comprises also the enormous group of regulatory RNA, which has become the focus of tumor research in the last years. MicroRNA (miRNA) and small nucleolar RNA (snoRNA) are two representatives of non-coding, but regulatory RNA. Both RNA species are described to be involved in the carcinogenesis of several tumors (Dong, Guo et al. 2009; Mocellin, Pasquali et al. 2009; Mourtada-Maarabouni, Pickard et al. 2009).

1.6.1 microRNA

microRNA (miRNA) is an evolutionarily conserved, small, non-coding RNA, which is functionally located between mRNA and protein by regulating the gene expression on the post transcriptional level. The biogenesis of the only approximately 22 nucleotides (nt) short miRNA is biphasic, located both in the nucleus and the cytoplasm (Grosshans and Filipowicz 2008). First, in the nucleus the primary transcript, called primary miRNA (pri-miRNA), is transcribed by the polymerase II (Lee, Kim et al. 2004). The pri-miRNA contains a stemloop structure which is recognized by the ribonuclease complex Drosha and its partner DGCR8. The Drosha and DGCR8 RNase III complex processes the pri-miRNA into the hairpin structured precursor miRNA (pre-miRNA) by cropping (Czech and Hannon 2011). The pre-miRNA is then exported into the cytoplasm via the nuclear transport receptor exportin 5 and further cleaved by the ribonuclease Dicer along with TARBP2 into the double stranded, approximately 22 nt short miRNA (Czech and Hannon 2011). At this state the miRNA

consists of the guide strand, which represents the future mature miRNA, and the passenger strand. The miRNA double strand is separated by Argonaute proteins. The passenger strand is generally degraded, whereas the mature miRNA is incorporated into the Argonaute protein containing RNA-induced silencing complex (RISC) (Bartel 2009). The RISC-miRNA complex then incorporates the target mRNA molecules and facilitates the function of the miRNA. The specific miRNA targeting is dependent on the complementarity between the 'seed' sequence of the miRNA and the 'seed-match' sequence of the target mRNA. The 'seed' sequence is defined as the positions 2 to 8 from the 5' end of the miRNA, whereas the 'seed-match' sequence is usually located in the 3' untranslated region of the target mRNA (Bartel 2009; Lujambio and Lowe 2012). Watson-Crick base-pairing enables the first recognition between the miRNA and the target mRNA and leads depending on the grade of complementarity to degradation of target mRNA (perfect complementarity) or translational repression (imperfect complementarity) (Bartel 2009). Both result in a negative expression regulation and therefore a decreased level of protein encoded on the target mRNA.

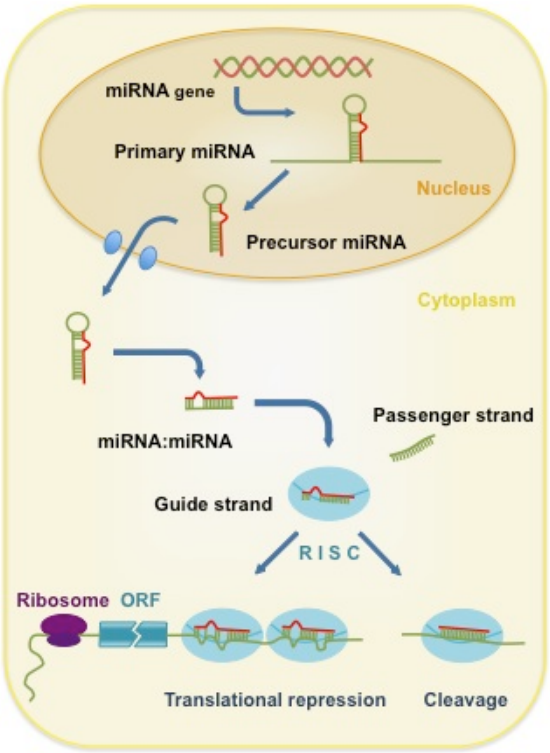

Figure 2: Biogenesis and function of miRNA (based on Giraldez)

It is assumed that more than 60 % of protein-coding genes are regulated by miRNA (Esteller 2011). Besides the aforementioned most common mechanisms of miRNA there are also descriptions of unexpected functions, e.g. binding to target genes and even enhancing gene expression (Stark, Lin et al. 2007; Orom, Nielsen et al. 2008). The regulatory network of miRNA is very complex. Single miRNAs can target multiple mRNA species and single mRNA

molecules on the other hand can be regulated by several miRNAs (Hon and Zhang 2007). Furthermore, miRNA regulation affects not only targets in linear pathways but also joining points between different circuits (Rottiers, Najafi-Shoushtari et al. 2011).

miRNA plays a crucial role in physiological processes like cell differentiation, proliferation, and apoptosis (Cheng, Byrom et al. 2005; Mendell 2005). Furthermore, miRNA is also involved in pathological conditions, since an altered miRNA expression pattern can be recognized in neurological, cardiovascular and developmental diseases, metabolic disorders and carcinogenesis (van Rooij, Sutherland et al. 2006; Kim, Inoue et al. 2007; Friedman and Avraham 2009; Mocellin, Pasquali et al. 2009; Xie, Lim et al. 2009). Its regulatory function in cell differentiation, proliferation and apoptosis emphasizes the role of miRNA in carcinogenesis, due to the fact that a deregulation of these physiological processes can lead to tumor development. Furthermore, an impact on tumor progression by miRNA is indicated by the finding that more than 50 % of human miRNA genes map in tumor associated genomic regions or within fragile sites of chromosomes (Calin, Sevignani et al. 2004).

Tumor related miRNAs are called oncomirs. Oncomirs are thought to function as oncogenes and tumor suppressors, respectively (Esquela-Kerscher and Slack 2006; Zhang, Pan et al. 2007). The expression of oncogenic oncomirs may therefore increase, and the expression of tumor suppressor miRNA may decrease in the course of tumor development. However, some oncomirs do not exclusively function as either oncogenes or tumor suppressors, but instead exhibit both properties dependent on the stage of tumor development and type of tumor (Rossi, Sevignani et al. 2008). Nevertheless, in most cases the specific function of particular oncomirs remains elusive. For prognostic and diagnostic use, it is of immense interest, if particular oncomirs do function as biomarkers for specific steps in carcinogenesis like cell invasion or metastatic spread.

The role of miRNA in tumorigenesis is often investigated by miRNA expression profiling comparatively between the non-neoplastic tissue and tumors that are derived from it. The B-cell chronic lymphocytic leukemia was the first tumor type for which an associated deregulated miRNA expression was observed (Calin, Dumitru et al. 2002). To date, an altered miRNA expression could be found in various other tumor types. Colorectal, prostate and cervical cancer show a down-regulation of miR-143 and miR-145, whereas in glioblastomas and breast cancer an up-regulation of miR-21 is observed. In human breast cancer, the first study focusing on miRNA expression in human patients and human breast cancer cell lines by Iorio and colleagues identified 29 miRNA species to be differentially expressed (Iorio, Ferracin et al. 2005). Wang and colleagues investigated the miRNA expression comparatively between breast cancer tissue and adjacent non-neoplastic tissue

(Wang, Zheng et al. 2010). These and other studies show both an up-regulation and down-regulation of certain miRNA species in breast cancer tissue compared to non-neoplastic tissue.

1.6.2 snoRNA

Another class of small, non-coding RNA is represented by the small nucleolar RNA (snoRNA). This regulatory RNA facilitates the site-specific modification of nucleotides in target RNA species (Mattick and Makunin 2005). Two main types of modifications occur, which are directed by two large families of snoRNAs: the box C/D snoRNA family guides 2-*O*-ribose methylation and the box H/ACA family, whose members direct pseudouridylation (Balakin, Smith et al. 1996; Bachellerie and Cavaille 1997; Kiss 2002). In each family, one snoRNA guides one or at most two modifications. To facilitate a regulation of target RNA, the guide snoRNA forms a specific duplex with the target RNA, whereas the actual catalytic process is conducted by a common enzyme, methylase or pseudouridine synthase, which is associated with the snoRNA (Bachellerie, Cavaille et al. 2002). The target RNA sequence is recognized by antisense interaction through the formation of a guide RNA duplex. Although the size of snoRNA ranges between 60 and 300 nt, only a short sequence is accountable for target recognition via one or two appropriate antisense elements (Mattick and Makunin 2005).

Initially, the function of snoRNA was assumed to be restricted to the modification of ribosomal RNA (rRNA) in ribosome biogenesis. However, recent studies show that both the complexity of the snoRNA families and the diversity of their targets have been underestimated. In addition to rRNA, other cellular RNA species are found to be targeted by new identified members of the snoRNA families like spliceosomal small nuclear RNA (snRNA) in vertebrates, transfer RNA (tRNA) in Archaea and potentially even eukaryotic mRNA. Besides snoRNAs with known targets there is a group of so called orphan snoRNA with unknown target (Bachellerie, Cavaille et al. 2002; Kiss, Jady et al. 2004).

Until recently, snoRNAs were thought to be suitable endogenous controls for miRNA real time RT-PCR, because of both their constitutive expression over diverse tissues and the miRNA-like properties like RNA stability and size (Davoren, McNeill et al. 2008; Carlsson, Helenius et al. 2010; Chang, Mestdagh et al. 2010; Brattelid, Aarnes et al. 2011). However, in the last years it could be shown, that snoRNA expression indeed is tissue-specific and furthermore specifically deregulated in pathological conditions like prostate and breast cancer (Dong, Rodriguez et al. 2008; Dong, Guo et al. 2009; Mourtada-Maarabouni, Pickard et al. 2009). Chang and colleagues provided the first indication for the influence of snoRNA

on carcinogenesis. They could show a tissue dependent expression of four human snoRNA species (h5sn1, h5sn2, h5sn3, and h5sn4). Furthermore, the expression of h5sn2 was drastically decreased in meningiomas relative to normal brain (Chang, Lin et al. 2002). Mourtada-Maarabouni and colleagues could draw an association between snoRNA expression and breast cancer. The non-coding growth arrest-specific transcript 5 (*GAS5*) gene, which can induce cell cycle arrest and apoptosis in breast cancer cell lines, encodes multiple snoRNAs in its introns. The expression level of *GAS5* transcripts was significantly decreased in breast cancer tissue compared to adjacent normal breast tissue. These findings led to the assumption that the related snoRNA U50 may act as a tumor suppressor in the carcinogenesis of breast cancer (Mourtada-Maarabouni, Pickard et al. 2009). The study of Dong and colleagues emphasized the role of U50 in tumor development and progression (Dong, Guo et al. 2009). The emerging evidence for a role of snoRNA in tumor development challenges the appropriateness of snoRNAs as reference genes. Furthermore, the question is raised, if formerly used reference snoRNAs are actually deregulated in breast cancer or other tumor types.

1.6.3 Quantification methods of small RNA species

One essential step for the investigation of the impact of small non-coding RNA species on various pathogenic conditions represents the detection of their expression. In recent years, several quantification methods have been developed and adopted successfully in miRNA profiling. Most of these assays are based on hybridization between complementary nucleotides of the target miRNA and a capture strand of nucleic acid. The hybridization event must be translated into a measurable signal like electrochemical detection, fluorescence or bioluminescence intensity. Hybridization methods can be classified into solid-phase and solution-phase methods. In the solid-phase, the target miRNA hybridizes with a capture probe that is bound or absorbed to a surface, whereas in solution phase, the target hybridization takes place in solution. The advantages of the solution-phase methods are the applicability for *in vivo* detection, high sensitivity and mostly short analysis times. In contrast, the solid-phase methods are adaptable to high-throughput screening, but inappropriate for *in vivo* analysis (Cissell and Deo 2009).

Representatives for solid-phase methods are northern blotting, microarray, electrochemical detection via oxidation of miRNA-ligated nanoparticles, and the bioluminescent detection method. In contrast, examples for solution-phase methods are bioluminescent protein reassembly, bioluminescence resonance energy transfer (BRET)-based detection, fluorescence correlation spectroscopy, and reverse transcriptase polymerase chain reaction (RT-PCR). Until recently, northern blotting was assumed to be the gold standard for miRNA

quantification. However, drawbacks of this method are time-consume and limited sensitivity (Cissell and Deo 2009; Li and Ruan 2009). The sensitivity of northern blotting can be improved by the use of locked nucleic acid (LNA)-probes (Varallyay, Burgyan et al. 2008). However, for high-throughput analyses cDNA microarrays are more appropriate than northern blotting (Li and Ruan 2009). But the high sample numbers are accompanied by high costs. The advantages of the electrochemical and bioluminescent detection are their high sensitivity. Nevertheless, both methods are holding measuring inaccuracies for signal decrease or increase (Cissell and Deo 2009). Similar measuring inaccuracies are held by the bioluminescent protein reassembly method and BRET-based detection, both solution phase methods with short analyses times and the potential for *in vivo* measurements (Cissell, Campbell et al. 2008; Cissell, Rahimi et al. 2008). In the fluorescence correlation spectroscopy method, miRNAs hybridize with their fluorophore-labeled targets. The hybridization events are countable by a fluorescent signal. This method is very sensitive and rapid and even allows the differentiation between single base mismatches (Neely, Patel et al. 2006; Cissell and Deo 2009). However, the required use of an external excitation source reduces the sensitivity of this technique.

Besides northern blotting and cDNA microarray, both solid phase methods, reverse transcriptase polymerase chain reaction (RT-PCR) represents another popular miRNA detection method. This solution phase method can be applied for *in vivo* measurements and it facilitates the quantification of small amounts of miRNA (Cissell and Deo 2009). However, the small size of mature miRNA and the sequence similarity within the different miRNA families pose specific challenges for primer design and overall specificity of the PCR assay. Moreover, the heterogeneous GC content of miRNAs leads to variable melting temperatures against miRNA specific primers (Benes and Castoldi 2010). In the last years, several modifications for RT-PCR have been established. Chen and colleagues developed a stem-loop RT-PCR (Chen, Ridzon et al. 2005). In this assay, a stem-loop RT-primer is applied for the reverse transcriptase reaction. The advantages of the stem-loop RT-primer are first an increase in specificity due to discriminating similar miRNA species by annealing a RT priming sequence to the 3' end of the mature miRNA. Furthermore, the double-stranded stem structure prevents non-specific hybridization of the RT-primer to miRNA precursors or other long RNA species. Second, the base stacking of the stem also increases the stability of miRNA-DNA-duplexes and thus enhances the efficacy of the reverse transcriptase reaction. Third, cDNA reverse transcribed with use of a stemloop RT-primer is extended in contrast to conventional transcribed miRNA. An extension of the RT product in turn eases the PCR primer design. The loop structure can comprise the sequence of the reverse PCR primer, whereas the forward PCR primer can be designed according to the miRNA sequence itself. Furthermore, an extended PCR template actually enables the use of hybridization probe

based PCR assays besides fluorescent dye based PCR methods (Schmittgen, Lee et al. 2008).

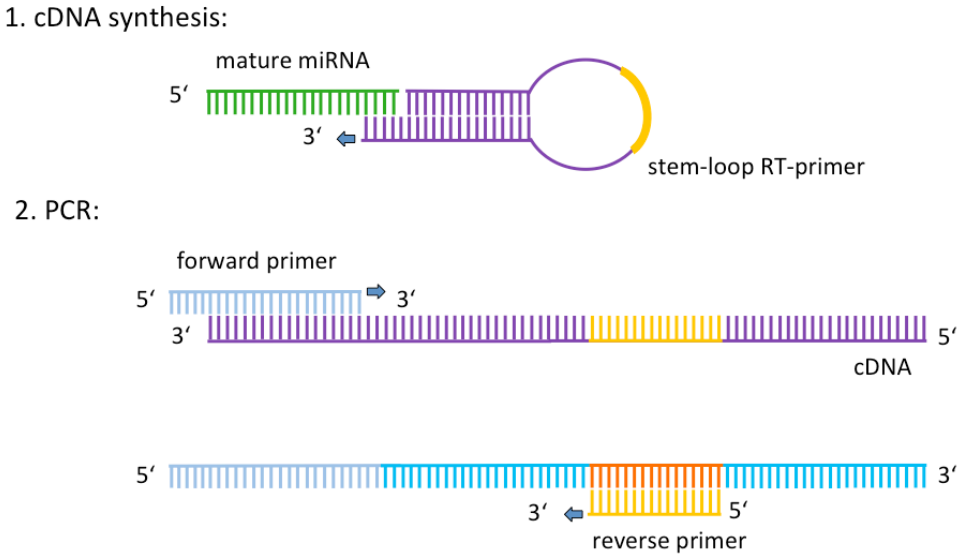

Figure 3: Schematic description of the stem-loop RT-PCR
(based on Schmittgen, Lee et al. 2008)

Nevertheless, a sensitive and particularly canine specific miRNA quantification method was still lacking at the time of this study. The investigation of the malignancy dependent expression pattern of miRNAs and snoRNAs in neoplastic canine tissue requires a reproducible and robust, canine specific detection method for accurate quantification of both miRNA and snoRNA. Several similarities suppose CMT to prove an ideal model for human breast cancer: both CMT and human breast cancer occur spontaneously, their biological behavior features local invasion, lymph node metastasis as well as distant metastasis, furthermore they show similar antigenetic phenotypes (Mottolese, Morelli et al. 1994; MacEwen 1996; Vail and MacEwen 2000). These findings lead to the assumption that oncomirs involved in the tumorigenesis of mammary cancer may be conserved between humans and dog (Boggs, Wright et al. 2008).

2 AIMS AND HYPOTHESIS

One of the greatest challenges in the diagnosis of CMT is the differentiation between non-metastasizing and metastasizing carcinomas prior to evident metastatic spread for reliable prognosis. It is still ambiguous, if the ability for forming metastases is a feature, which the tumor develops in the course of malignant progression or if the potential for metastatic spread is inherent in a particular tumor from the very beginning of tumor development. In both cases the classical diagnostic tools reach their limits and it is of immense interest to understand, which essential molecular factors are responsible for the neoplastic transformation of cells, the malignant progression and metastatic behavior. According to the literature summarized in the introduction part of this doctoral thesis, differentially expressed miRNA and snoRNA represent potential biomarkers for cancer diagnosis.

2.1 Main hypothesis

Particular miRNA and snoRNA species are specifically expressed in canine mammary tumors at different stages of malignancy. Thus, their expression pattern allows a differentiation between non-neoplastic mammary gland and neoplastic mammary tissue, benign tumors and malignant tumors as well as between non-metastasizing and metastasizing carcinomas.

To test this hypothesis the following objectives were set for our project:

2.2 First aim

Establishment of a canine specific real-time PCR assay with suitable endogenous controls for accurate quantification of miRNA and snoRNA

2.3 Second aim

Correlation of the expression pattern of 16 miRNAs and 4 snoRNAs with canine mammary gland and canine mammary tumors at different stages of malignancy

3 RESEARCH PUBLICATIONS IN PEER-REVIEWED JOURNALS

3.1 Molecular quantification of canine specific microRNA species

Authors: von Deetzen M-C, Schmeck B, Gruber AD, Klopfleisch R
Year: 2013
Journal: Research in Veterinary Science 95 (2013) 562–568

DOI: 10.1016/j.rvsc.2013.06.012

Declaration of own portion of work in the research publication:

Contributions of M-C von Deetzen: Independent design, preparation, completion and evaluation of all experiments with exception of sequence analysis of PCR products. Independent subsequent creation of the entire manuscript.

Contributions of other authors: All coauthors served as counseling team regarding design and interpretation of experiments and participated in subsequent compilation of parts of the manuscript relating to these analyses.

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3.2 Malignancy Associated MicroRNA Expression Changes in Canine Mammary Cancer of Different Malignancies

Authors: von Deetzen M-C, Schmeck B, Gruber AD, Klopfleisch R
Year: 2014
Journal: ISRN Veterinary Science Volume 2014 (2014), Article ID 148597, 5 pages

DOI: 10.1155/2014/148597

Declaration of own portion of work in the research publication:

Contributions of M-C von Deetzen: Independent design, preparation, completion and evaluation of all experiments as well as independent subsequent creation of the entire manuscript.

Contributions of other authors: All coauthors served as counseling team regarding design and interpretation of experiments and participated in subsequent compilation of parts of the manuscript relating to these analyses.

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Research Article

Malignancy Associated MicroRNA Expression Changes in Canine Mammary Cancer of Different Malignancies

Marie-Charlotte von Deetzen,[1] Bernd T. Schmeck,[2] Achim D. Gruber,[1] and Robert Klopfleisch[1]

[1] *Institute of Veterinary Pathology, Freie Universität Berlin, Robert-von-Ostertag-Straße 15, 14163 Berlin, Germany*
[2] *Molecular Pulmonology, German Center for Lung Research, Philipps Universität Marburg, Hans-Meerwein-Straße 2, 35043 Marburg, Germany*

Correspondence should be addressed to Robert Klopfleisch; robert.klopfleisch@fu-berlin.de

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MicroRNA has been suspected to be generally involved in carcinogenesis since their first description. A first study supported this assumption for canine mammary tumors when miRNA expression was compared to normal gland. The present study extends these results by comparing the expression of 16 microRNA (miRNA) and 4 small nucleolar RNA (snoRNA) in tumors of different malignancy, for example, adenomas, nonmetastasizing and metastasizing carcinomas as well as lymph node metastases, with each other and with normal mammary gland. All neoplastic tissues differed in their miR-210 expression levels from normal gland. While metastatic cells differed in their expression of mir-29b, miR-101, mir-125a, miR-143, and miR-145 from primary tumors, the comparison of miRNA expression in primary tumors of different malignancy failed to reveal significant differences except for a significant downregulation of mir-125a in metastasizing carcinomas when compared to adenomas.

1. Introduction

MicroRNA (miRNA) is an evolutionarily conserved, noncoding, but regulatory RNA species of approximately 22 nucleotides in length. It plays a crucial role in various physiological and pathological processes by regulating gene expression posttranscriptionally. miRNA binds to messenger RNA (mRNA) and thereby induces a sequence-depending mRNA degradation or translational repression [1–3]. A deregulation of miRNA is associated with a wide variety of pathologic states including carcinogenesis [4]. Nevertheless, in many cases the specific function of individual miRNA species is still unknown. For instance, miR-10b has been identified as a tumor suppressor which prevents human breast cancer development but also as an oncogene which initiates breast cancer invasion and metastasis [5]. Several miRNA species have been identified to be involved in human breast cancer development including miR-21, miR-145, and miR-210 [6–8]. In veterinary medicine, only a single study is available on miRNA expression in canine mammary tumors. Boggs et al. [7] compared the expression levels of ten miRNA species in malignant mammary tumors and normal canine mammary gland and found a significant deregulation of miR-21, miR-29b, let-7f, miR-15a, and miR-16 in the tumors.

In the present study, we expand these recent findings on the impact of miRNA deregulation on canine mammary tumors by asking for differences in expression levels of four small nucleolar RNA (snoRNA) and 16 canine miRNA with known relevance for human and canine mammary tumor development in tissue samples of normal mammary gland, adenomas, metastasizing, and nonmetastasizing canine mammary carcinomas as well as lymph node metastases.

2. Materials and Methods

Mammary gland tissues including regional lymph nodes from 30 dogs submitted to the Department of Veterinary Pathology of the Freie Universität Berlin were included in the study. Clinical data of the dogs included breed, age, and

location of tumors. All tissue samples were freshly frozen not later than 30 min after surgical excision and stored at −80°C until further use. Directly adjacent tissue samples were also fixed in 4% neutral buffered formalin, routinely processed stained with haematoxylin and eosin (HE) for histologic examination. Ten simple mammary adenomas, ten simple carcinomas without evidence of tumor cells in the regional lymph node, and ten simple carcinomas with regional lymph node metastases as well as normal mammary gland tissues of ten dogs were selected and macrodissected for this study to assure a tumor content of more than 80%. The fifth sample group contained laser microdissected lymph node metastases from five dogs with simple, metastasizing mammary carcinoma.

The study was approved by the local animal welfare officer. Surgical resection of the tumors was part of the therapy according to the welfare of the animals and to the state of the art of medical science under full anesthesia. The study therefore had no influence on the common diagnostic or therapeutic measures usually applied on animals with mammary gland tumors and inclusion into the study did not induce any additional treatments, pain, or discomfort-inducing manipulations during the entire study.

Total RNA was extracted as previously described [9, 10]. The lymph node metastases were microdissected from cryostat sections by laser capture microdissection as previously described [11]. All miRNA and snoRNA species were elongated prior to cDNA synthesis by universal poly-A-tailing and amplified by quantitative reverse transcription PCR as previously described [9]. Housekeeping genes were selected from the complete set of genes analyzed using both geNorm (version 3.4) and NormFinder (version 20) algorithms [12, 13]. The most stably expressed RNA species were determined by calculating the gene expression stability measure value (M) using the geNorm tool as previously described [12]. Stepwise exclusion of the least stable genes identified miR-155, let-7f, and miR-181b as the three most stably expressed genes. In a second approach NormFinder analysis was performed [13] and identified miR-181b, U44, and U48 as the three most stable reference genes. Considering both the results of the geNorm analysis and the NormFinder algorithm miR-181b, miR-155, and U44 were used for data normalization. Consequently, relative expression levels of the miRNA and snoRNA species were determined using the $2^{-\Delta\Delta Ct}$ method [14]. Due to the different efficiencies of the PCR assays, the actual efficiencies were used as the base of the exponential amplification function for calculation. Statistical significance of differences in miRNA and snoRNA expression levels was evaluated using the IBM SPSS Statistic 20 software. The results in the different tissue groups were statistically compared using the parametric ANOVA analysis. A $P \leq 0.05$ was considered statistically significant.

3. Results

Comparison of the expression levels of 16 miRNA and four snoRNA leads to the identification of statistically different expression levels of nine miRNA and one snoRNA in 27 of the 400 comparisons. Of these, microdissected tumor metastases differed most often from other tissue types. miR-101, miR-143, and miR-145 were differently expressed in metastases when compared to all other tissues. miR-29b differed in its expression level between metastases and all tumor tissue samples except normal mammary gland. miR-125a expression only differed between metastases and metastasizing carcinomas primary sites (Table 1, Figure 1). The comparison of the primary tumors of different malignancies identified a difference in miR-125a expression between metastasizing carcinoma and adenoma as the only significant difference (Table 1, Figure 1). All other differences in expression were restricted to comparisons between normal mammary gland and neoplastic tissues. miR-21, miR-143, miR-194, miR-203, miR-210, and the snoRNA U24 differed in their expression levels between normal gland and neoplastic tissues (Table 1, Figure 1). Of note, only miR-125a and the snoRNA U24 showed a decreased expression in the primary tumors when compared to normal gland or adenomas while all other significantly regulated miRNA had an upregulation in benign and malignant primary tumors (Table 1). This was in contrast to the observation in metastases which, except for miR-125a and miR-210, had a general downregulation of miRNA when compared to all other tissues (Table 1).

miR-210 was the only miRNA which allowed the differentiation of normal mammary gland from all neoplastic tissues. In addition, miR-210 was the only miRNA with a continuous significant increase in expression levels between normal mammary gland and all tumor groups and the metastases with expression differences of 7.01-fold between adenomas and normal gland, 10.41-fold between nonmetastasizing carcinomas and normal gland, 10.72-fold between metastasizing carcinomas and normal gland, and 19.63-fold between metastases and normal gland (Table 1).

U24 was the only snoRNA with a significant difference in expression. The expression difference was 0.38-fold between the metastasizing carcinoma and the normal mammary gland but was not significantly altered in any other comparison. miR-9, miR-10b, miR-15a, miR-16, miR-125b, miR-136, and let-7f as well as the snoRNA U66, Z30, and U48 had no significant difference in expression between any of the tissues analyzed.

4. Discussion

The aim of the present study was the identification of malignancy associated miRNA expression to discover potential malignancy marker and to further elucidate aspects of the molecular carcinogenesis and gene expression associated with metastatic behavior of canine mammary tumors [15]. To this end, the expression of 16 miRNA and four snoRNA was compared between normal mammary gland, adenomas, carcinomas, and metastases. Only nine of the miRNA species and one snoRNA tested were significantly differently expressed between the diverse tissues analyzed.

Interestingly, miR-210 was significantly overexpressed in all neoplastic tissues when compared to normal mammary gland. Previous work described miR-210 as a "hypoxamir" which is upregulated in hypoxic tissues by HIF-1 action and mediates a metabolic adaptation to anaerobic conditions [16].

Table 1: miRNA and snoRNA expression differences between mammary tissues at different stages of malignancy and normal gland.

	Normal	Adenoma	Nonmetastasizing carcinoma	Metastasizing carcinoma	Metastasis	Regulation
Normal		miR-203 (4.37) miR-210 (7.01)	miR-143 (2.70) miR-21 (3.18) miR-210 (10.41) miR-194 (4.44)	miR-21 (5.05) miR-210 (10.72)	miR-210 (19.63)	Up
				U24 (0.38)	miR-143 (0.04), miR-145 (0.02), miR-101 (0.12)	Down
Adenoma						Up
				miR-125a (0.40)	miR-143 (0.02), miR-145 (0.02), miR-101 (0.05), miR-29b (0.14)	Down
Nonmetastasizing carcinoma						Up
					miR-143 (0.02), miR-145 (0.01), miR-101 (0.08), miR-29b (0.18)	Down
Metastasizing carcinoma					miR-125a (4.91)	Up
					miR-143 (0.06), miR-145 (0.04), miR-101 (0.13), miR-29b (0.18)	Down
Metastasis						Up Down

In brackets (): fold change in expression difference.

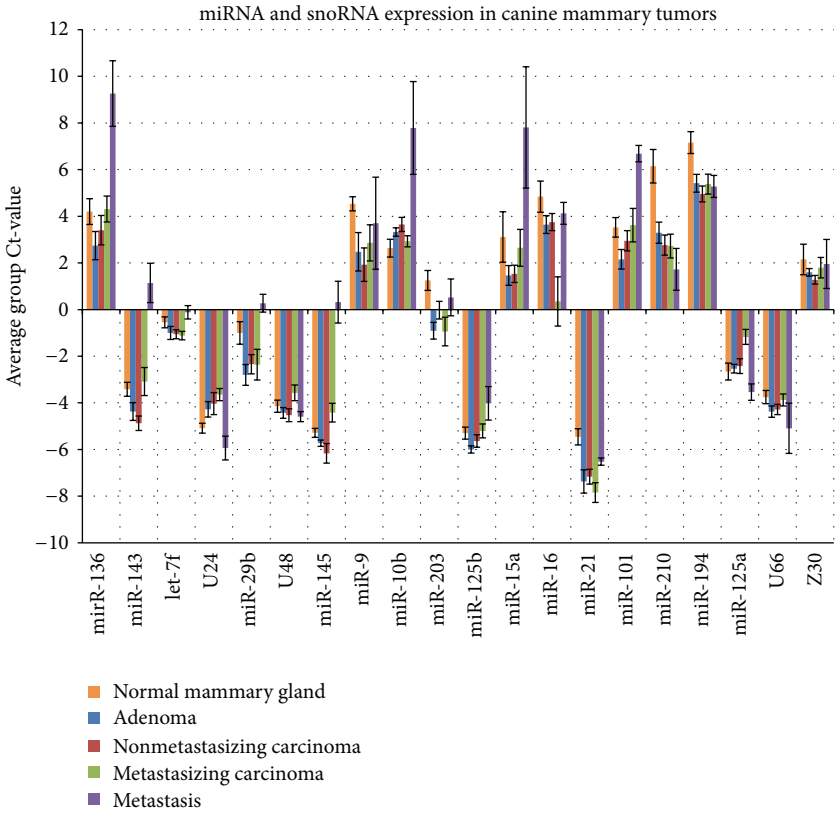

Figure 1: miRNA and snoRNA expression levels in normal gland, adenoma, nonmetastasizing carcinoma, metastasizing carcinoma, and lymph node metastasis. Ct-values are average values of expression levels of the tissue groups normalized to three housekeeping genes.

The continuous upregulation of miR-210 over the course of malignant progression may therefore be caused by an increasing hypoxia in the developing tumor mass. miR-210 overexpression has also been associated with the formation of capillary-like structures [17]. It can thus be hypothesized that miR-210 may indirectly promote metastasis by triggering angiogenesis in neighboring cells [18]. Another four miRNA, miR-21, miR-143, miR-194, and miR-203, were significantly increased in at least one mammary tumor group compared to normal mammary gland and therefore matched the definition of an oncogenic miRNA. While this disease association is new for miR-143 and miR-194, a similar oncogenic function has been suggested for miR-203 [19] in mammary cancer and for miR-21 in several other tumors including human breast cancer but has to be determined yet [20].

The general lack of differences in miRNA expression between primary tumors at different stages of malignancy was intriguing. Only miR-125a was significantly differently expressed between metastasizing carcinoma and adenoma. This was in contrast to the numerous miRNA differentially expressed in metastatic tumor cells in the lymph nodes. This difference may be caused by the different preparation of metastatic tumor cells by laser microdissection technology due to the otherwise high contamination of metastases tissue samples by lymphatic cells. The macrodissected samples of normal gland and the primary tumors in contrast also contained, although at a minimal portion, cells of the tumor stroma. These cells may have contributed to the recorded downregulation of the four miRNA in microdissected metastases when compared to macrodissected primary tumors and normal mammary gland, respectively.

5. Conclusions

In conclusion, canine mammary tumors at different stages of malignancy significantly differed from normal gland in the expression of seven miRNA and one snoRNA species. miRNA and snoRNA expression however failed in most cases to discriminate primary tumors at different stages of malignancy.

Conflict of Interests

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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References

[1] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[2] V. Ambros, "The functions of animal microRNAs," *Nature*, vol. 431, no. 7006, pp. 350–355, 2004.

[3] A. M. Cheng, M. W. Byrom, J. Shelton, and L. P. Ford, "Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis," *Nucleic Acids Research*, vol. 33, no. 4, pp. 1290–1297, 2005.

[4] B. Zhang, X. Pan, G. P. Cobb, and T. A. Anderson, "MicroRNAs as oncogenes and tumor suppressors," *Developmental Biology*, vol. 302, no. 1, pp. 1–12, 2007.

[5] L. Ma, J. Teruya-Feldstein, and R. A. Weinberg, "Tumour invasion and metastasis initiated by microRNA-10b in breast cancer," *Nature*, vol. 449, no. 7163, pp. 682–688, 2007.

[6] M. V. Iorio, M. Ferracin, C.-G. Liu et al., "MicroRNA gene expression deregulation in human breast cancer," *Cancer Research*, vol. 65, no. 16, pp. 7065–7070, 2005.

[7] R. M. Boggs, Z. M. Wright, M. J. Stickney, W. W. Porter, and K. E. Murphy, "MicroRNA expression in canine mammary cancer," *Mammalian Genome*, vol. 19, no. 7-8, pp. 561–569, 2008.

[8] L. Hong, J. Yang, Y. Han, Q. Lu, J. Cao, and L. Syed, "High expression of miR-210 predicts poor survival in patients with breast cancer: a meta-analysis," *Gene*, vol. 507, no. 2, pp. 135–138, 2012.

[9] M. C. von Deetzen, B. Schmeck, A. D. Gruber, and R. Klopfleisch, "Molecular quantification of canine specific microRNA species," *Research in Veterinary Science*, vol. 95, no. 2, pp. 562–568, 2013.

[10] R. Klopfleisch, D. Lenze, M. Hummel, and A. D. Gruber, "The metastatic cascade is reflected in the transcriptome of metastatic canine mammary carcinomas," *Veterinary Journal*, vol. 190, no. 2, pp. 236–243, 2011.

[11] R. Klopfleisch, P. Klose, and A. D. Gruber, "The combined expression pattern of BMP2, LTBP4, and DERL1 discriminates malignant from benign canine mammary tumors," *Veterinary Pathology*, vol. 47, no. 3, pp. 446–454, 2010.

[12] J. Vandesompele, K. de Preter, F. Pattyn et al., "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes," *Genome Biology*, vol. 3, no. 7, pp. 34.1–34.11, 2002.

[13] C. L. Andersen, J. L. Jensen, and T. F. Orntoft, "Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets," *Cancer Research*, vol. 64, no. 15, pp. 5245–5250, 2004.

[14] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T))," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[15] R. Klopfleisch, H. von Euler, G. Sarli, S. S. Pinho, F. Gärtner, and A. D. Gruber, "Molecular carcinogenesis of canine mammary tumors: news from an old disease," *Veterinary Pathology*, vol. 48, no. 1, pp. 98–116, 2011.

[16] R. Kulshreshtha, M. Ferracin, S. E. Wojcik et al., "A microRNA signature of hypoxia," *Molecular and Cellular Biology*, vol. 27, no. 5, pp. 1859–1867, 2007.

[17] P. Fasanaro, Y. D'Alessandra, V. di Stefano et al., "MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand ephrin-A3," *Journal of Biological Chemistry*, vol. 283, no. 23, pp. 15878–15883, 2008.

[18] H. Cui, S. Grosso, F. Schelter, B. Mari, and A. Krüger, "On the pro-metastatic stress response to cancer therapies: evidence for a positive co-operation between TIMP-1, HIF-1alpha, and miR-210," *Frontiers in Pharmacology*, vol. 3, article 134, 2012.

[19] P. Ru, R. Steele, E. C. Hsueh, and R. B. Ray, "Anti-miR-203 upregulates SOCS3 expression in breast cancer cells and enhances cisplatin chemosensitivity," *Genes and Cancer*, vol. 2, no. 7, pp. 720–727, 2011.

[20] S. Zhu, M.-L. Si, H. Wu, and Y.-Y. Mo, "MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1)," *The Journal of Biological Chemistry*, vol. 282, no. 19, pp. 14328–14336, 2007.

4 CONCLUDING DISCUSSION

To enlighten the role of miRNA and snoRNA in the molecular carcinogenesis of canine mammary tumors (CMT) a canine specific real-time PCR protocol for the quantification of a set of 16 miRNAs and 4 snoRNAs was developed. While the snoRNA species were formerly used as housekeeping genes for PCR data normalization, the selected miRNA species were known to be deregulated in neoplastic mammary tissue versus non-neoplastic mammary gland in humans and dogs, respectively (Iorio, Ferracin et al. 2005; Boggs, Wright et al. 2008). We expanded these recent findings and compared the expression patterns of non-neoplastic mammary gland and CMT at different stages of malignancy. In this chapter, the hypothesis and aims of this study are discussed.

4.1 First aim: Establishment of a canine specific real-time PCR assay with suitable endogenous controls for accurate quantification of miRNA and snoRNA

The establishment of a canine specific real-time PCR assay implicates four steps. First, a reverse transcription method that is suitable for both miRNA and snoRNA was developed. Second, canine specific PCR primers were designed and several PCR variables were optimized for sensitive and specific quantification of miRNA and snoRNA. Third, suitable endogenous controls for normalization of the PCR data were compiled. Fourth, PCR products were subsequently sequenced to assure the specificity of the real-time PCR assay.

4.1.1 Reverse transcription

The first step for the establishment of a canine specific real-time PCR assay was the design of a reverse transcription method suitable for miRNA and snoRNA. The small size of miRNA makes the use of conventional primers for cDNA synthesis impracticable. Thus, prior to reverse transcription an elongation step was conducted with total RNA. To this end, two different methods were comparatively investigated.

First, total RNA was elongated by adding a poly-(A)-tail to the 3’ end of each RNA. This universal approach enables a simultaneous reverse transcription of a set of miRNAs and snoRNAs. In the next step, a stemloop structured oligo-(dT)-anchor was annealed to the 3’ end of the poly-(A)-tail. The oligo-(dT)-anchor contained the sequence of the universal reverse PCR primer in its loop. Finally, the tailed RNA was reverse transcribed.

Second, in the individual reverse transcription approach each miRNA and snoRNA species was individually elongated and reverse transcribed by using a specific reverse transcription primer (RT-primer). The last 5-6 nt of the 3' end of the RT-primer were complementary to the individual miRNA and snoRNA species, respectively. The first 44 nt of the RT-primer represent the same stemloop structure as the oligo-(dT)-anchor.

4.1.2 Quantitative real-time PCR assay

The subsequent SYBRGreen based real-time PCR was conducted with canine specific primers. Sequence information for the primer design was obtained from the mirBase data base version 17 for miRNA primers and from the Ensemble genome browser release 62 for snoRNA primers. The forward primers were designed specifically for each miRNA and snoRNA. Thus, the miRNA forward primers were designed with a linear 5′ overlap of 4-6 nt and covered all but the last 5-6 nt of the microRNA sequence. The snoRNA forward primers were similarly designed, with the exception of U66, for which only the first 5′ third of the snoRNA sequence was used due to the larger size of this snoRNA species. The reverse primer was universal, because its sequence was contained in the loop of the oligo-(dT)-anchor and specific RT-primer, respectively. PCR variables like annealing temperature and primer concentration were tested to identify the optimal PCR protocol. The comparison of specificity and sensitivity in miRNA and snoRNA detection of the universally and individually transcribed cDNA did not lead to any significant differences for the two approaches. Nevertheless, the advantages of a simultaneous cDNA synthesis were the lower costs, the smaller amounts of tissue required and the shorter protocol. On this account, the universal transcription approach was applied for malignancy dependent expression analyses of miRNA and snoRNA in CMT in the second aim of this thesis.

4.1.3 Stability of housekeeping gene expression

The normalization to one or more stably expressed endogenous control genes is essential for accurate determination of the relative expression levels of target genes. Endogenous control genes, also called housekeeping genes, have to be in line with certain criteria like similar size and stability as the target genes and constitutive expression over diverse tissues. Until recently, snoRNA was often used for the normalization of miRNA real-time PCR data. As mentioned before, current studies call the principal suitability of snoRNA as housekeeping genes in question (Peltier and Latham 2008; Mortarino, Gioia et al. 2010; Gee, Buffa et al. 2011). Particularly snoRNAs are described to be differently expressed in pathological conditions including breast cancer (Mourtada-Maarabouni, Pickard et al. 2009). For the identification of suitable housekeeping genes the expression stability of all miRNA and

snoRNA species was analyzed. To this end, raw expression levels obtained from the real-time PCR runs were used for both geNorm (version 3.4) and NormFinder (version 20) analyses. The results of the geNorm and NormFinder algorithms were the same for the separate analysis of all snoRNAs. The separate analysis of all miRNAs led to the same five most stably expressed genes with a mild variation in the order of the second most and third most stably expressed miRNA. Interestingly, the collective analysis of all miRNAs and snoRNAs with the NormFinder algorithm resulted in the same five most stably expressed miRNA species. In contrast, the collective analysis with the geNorm algorithm presented two miRNAs and three snoRNAs as the five most stably expressed genes. Over all analyses, two same genes were always within the five most stably expressed genes. These two genes represent miRNAs (miR-181b and miR-155). Considering both the results of geNorm and NormFinder analyses, two miRNA species (miR-181b, miR-155) and one snoRNA species (U44) were identified as suitable housekeeping genes and were used for PCR data normalization. In general, the combined analysis of miRNA and snoRNA expression stability identified an unexpected trend towards more stable expressed miRNA compared to snoRNA.

4.1.4 Sequence analysis of PCR products

The sequence analysis of the PCR products posed another challenge in establishing a real-time PCR assay for miRNA and snoRNA quantification. Conspicuously, no detailed description of a sequencing method could be found in the literature. The standard procedure of sequencing the purified approximately 88 base pairs (bp) short miRNA PCR products in commercial sequence laboratories was impossible. Sequencing of the snoRNA PCR products with an average length of 150 bp also failed in the majority of cases. Thus, not only the small size of the miRNA PCR products but also the containing stemloop structure seemed to influence the sequence analysis. Therefore, all PCR products were cloned by TOPO TA Cloning (TOPO TA Cloning Kit, Invitrogen) prior to sequence analysis. This procedure led to stable and specific sequencing results for both miRNA and snoRNA. Thereby, our canine specific real-time PCR assay could be assured, since all examined miRNA and snoRNA species were specifically amplified.

In conclusion, this study established an accurate and manageable, canine specific quantification assay for 21 miRNA and 5 snoRNA species. This SYBRGreen based, quantitative real time PCR assay allows a robust relative quantification of miRNA and snoRNA in canine tissue.

4.2 Second aim:
Correlation of the expression pattern of 16 miRNAs and 4 snoRNAs with canine mammary gland and canine mammary tumors at different stages of malignancy

The second objective of our study was the investigation of the malignancy dependent expression of miRNA and snoRNA in CMT. To this end our canine specific real-time PCR assay was conducted for a set of 16 miRNAs and 4 snoRNAs in non-neoplastic mammary gland and canine mammary tumors at different stages of malignancy. The CMT were histologically diagnosed and grouped into adenomas, non-metastasizing carcinomas, metastasizing carcinomas and lymph node metastases. The normalized expression data were subsequently compared between the different tissue groups as depicted in figure 4. The statistical analysis led to significant different expression levels in nine miRNAs and one snoRNA. The following sections will only respond to the significant expression results.

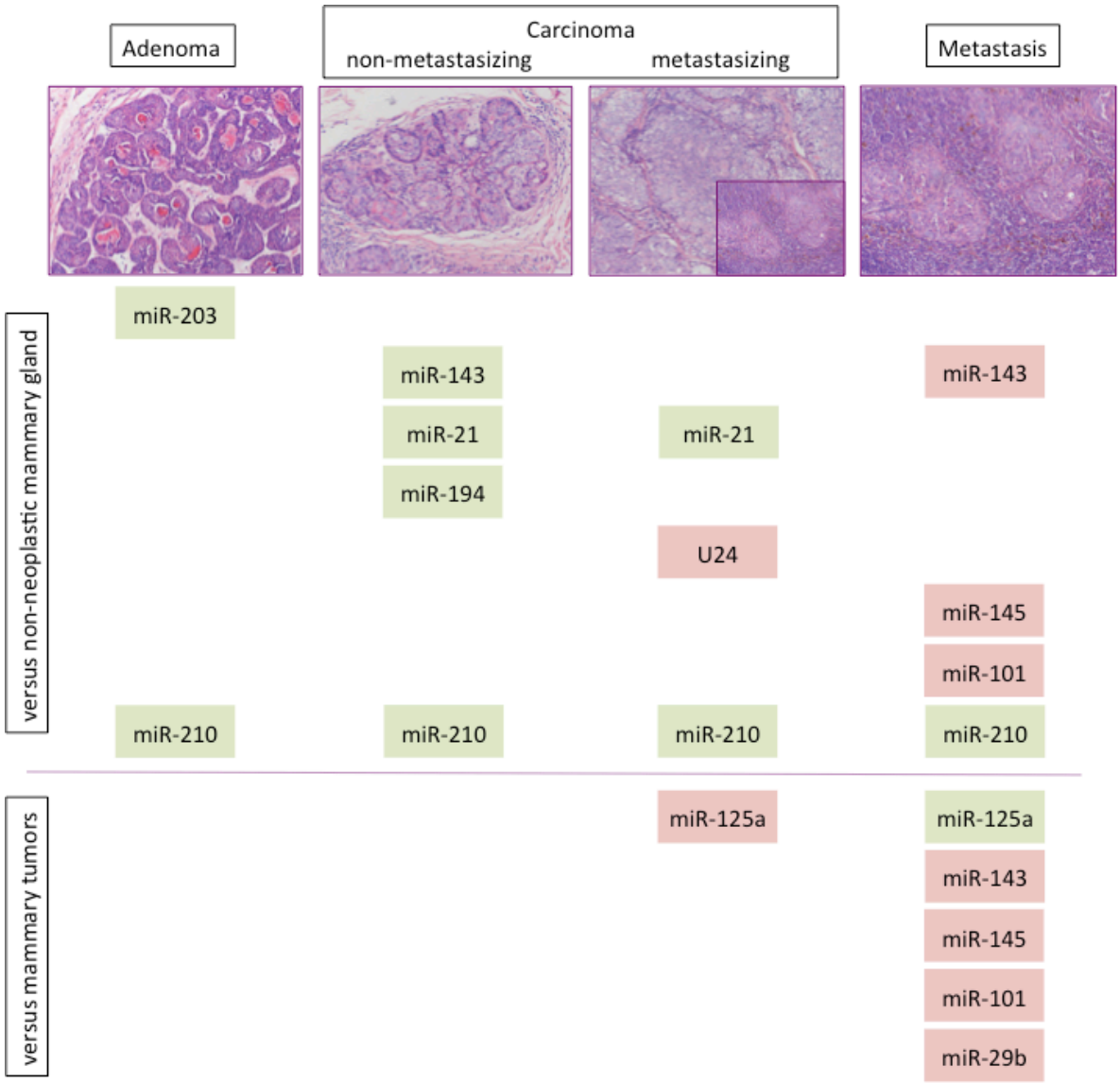

Figure 4: Differential regulation of miRNA and snoRNA in adenoma, non-metastasizing and metastasizing carcinoma and lymph node metastasis.
Green box = up-regulated expression. Red box = down-regulated expression.

4.2.1 Up-regulated expression

Compared to non-neoplastic mammary gland, the expression was increased in the primary tumors for five miRNAs (miR-203, miR-143, miR-21, miR-194, miR-210). One of these miRNAs (miR-210) showed also a significant up-regulation in the group of metastases versus normal mammary gland. Remarkably, only miR-125a showed a significantly increased expression in the metastases compared to primary tumors (metastasizing carcinomas). In particular, miR-210 was the only miRNA that allows the differentiation of non-neoplastic mammary gland to all other primary and metastatic tumor groups. Although the expression

increased continuously with the course of malignant progression from the adenoma over the carcinoma towards the metastasis, the expression between the tumor groups itself was not significantly different. An overexpression of miR-210 was found under hypoxic circumstances, a known factor of tumor microenvironment (Kulshreshtha, Ferracin et al. 2007). Thus, the continuous up-regulation of miR-210 expression over the course of malignant progression in our study may be caused by an increased hypoxia in the developing tumor mass. Furthermore, an increased expression of miR-210 could be associated with the formation of capillary like structures (Fasanaro, D'Alessandra et al. 2008). Therefore, it was hypothesized, that miR-210 may be involved in tumor growth and promotion of metastasis by triggering angiogenesis (Cui, Grosso et al. 2012). miR-21 showed an up-regulated expression in both malignant primary tumor groups compared to the normal mammary gland. An increased expression of miR-21 is described in various solid tumors (Garzon, Marcucci et al. 2010; Farazi, Spitzer et al. 2011). Furthermore, in breast cancer a correlation between miR-21 overexpression and advanced clinical stage, lymph node metastasis and poor prognosis could be found (Yan, Huang et al. 2008). An oncogenic function has also been suggested for mir-203 and miR-194 (Ru, Steele et al. 2011; Iizuka, Imaoka et al. 2013). All miRNAs that show an overexpression in neoplastic tissue compared to non-neoplastic mammary gland are potential oncogenic oncomirs.

4.2.2 Down-regulated expression

The only RNA species being down-regulated in primary tumors (metastasizing carcinomas) when compared with the non-neoplastic mammary tissue was the snoRNA U24. Remarkably, in the group of metastases the expression of four miRNAs (miR-143, miR-145, miR-101, miR-29b) was decreased compared to all primary tumors. Three of these four miRNAs showed also a decreased expression when compared to the non-neoplastic mammary tissue. miRNA species showing a decreased expression in neoplastic tissue versus non-neoplastic mammary gland are potential tumor suppressors. A current study identified miR-143 and miR-145 to repress synergistically the expression of ERBB3 in breast cancer leading to suppression of proliferation and invasion of tumor cells. Therefore, the miR-143/miR-145 cluster is suggested to function as a tumor suppressor in breast cancer (Yan, Chen et al. 2014). Wang and colleagues explored the effect of the down-regulated miR-101 in breast cancer cells. Via targeting the Janus kinase 2 miR-101 suppresses tumor cell proliferation and promotes apoptosis (Wang, Li et al. 2014).

A differentiation between primary tumors was only enabled by miR-125a. In detail, the expression of miR-125a was decreased in metastasizing carcinomas when compared to adenomas. miR-125 expression is described to be decreased in human breast cancer as

compared to non-neoplastic breast tissue (Iorio, Ferracin et al. 2005). Another study revealed that the expression of miR-125 inversely correlates with the HuR protein level in several breast carcinoma cell lines. Interestingly, the mRNA encoding the stress-induced RNA binding protein HuR is only targeted by miR-125a but not by miR-125b. This leads to decreased HuR protein levels and thus inhibition of cell growth and cell migration. These findings indicate miR-125a to function as a tumor suppressor in breast cancer (Guo, Wu et al. 2009). However, other studies provide conflicting results, since both members of miR-125 target the tumor suppressor p53 mRNA and thus modulate the apoptotic stress response in other mammalian cells (Le, Teh et al. 2009; Zhang, Gao et al. 2009). The expression of miR-125a seems to depend on the tissue type or developmental stage. This may explain why the expression of miR-125a increased again in the metastases when compared to the metastasizing carcinomas.

4.3 miRNAs as biomarkers for cancer diagnosis

The prognosis of a breast cancer patient is highly dependent on the stage of malignancy of the tumor and is even worse at the very moment when metastases occur. The differentiation between benign adenomas and malignant carcinomas can be performed by histologic biopsies. However, the histopathological diagnosis of carcinomas is often insufficient for prognosis particularly before metastases are histologically evident. The diagnosis of canine mammary tumors therefore lacks biomarkers which enable the differentiation of histologically defined carcinomas into non-metastasizing and metastasizing carcinomas.

Our study reports different miRNA expression patterns of major stages in malignant progression: adenoma, non-metastasizing carcinoma, metastasizing carcinoma and the endpoint metastasis. The expression of miR-21 and miR-210 was up-regulated in both metastasizing and non-metastasizing carcinomas versus non-neoplastic tissue. Recent studies could correlate miR-21 overexpression in human breast cancer with advanced clinical stage and lymph node metastasis (Yan, Huang et al. 2008). miR-210 expression increased continuously over the course of malignant progression. This hypoxia induced miRNA species is suggested to be involved in tumor growth and promotion of metastasis by triggering angiogenesis (Fasanaro, D'Alessandra et al. 2008; Cui, Grosso et al. 2012). In turn, the expression of U24 and miR-125a was decreased in metastasizing carcinomas but not in non-metastasizing carcinomas. To date, an association between U24 and forming metastases is not described. miR-125a allowed a differentiation between metastasizing carcinomas and adenomas as well as between metastasizing carcinomas and metastases, in which miR-125a expression increased again. Guo and colleagues suggested miR-125a to

target HuR and thus function as a tumor suppressor in human breast cancer (Guo, Wu et al. 2009). This supports our finding that miR-125a was overexpressed in metastasizing carcinomas. Other studies identified miR-125 to target the tumor suppressor p53 mRNA and therefore to function as an oncogenic oncomir (Le, Teh et al. 2009; Zhang, Gao et al. 2009). These conflicting results lead to the suggestion that the expression of miR-125a is dependent on the tissue type or developmental stage and might explain why the expression of miR-125a increased again in the metastases when compared to the metastasizing carcinomas.

An unambiguous biomarker for metastatic behavior, which allows the differentiation between non-metastasizing and metastasizing carcinomas, could not be identified in the investigated set of 16 miRNAs and 4 snoRNAs. However, this finding has to be interpreted in the context of malignant progression as a continuous process and the stroma-tumor cell interplay as discussed in the next section.

4.4 The stroma-tumor cell interplay

Recent studies have shown that certain features of carcinogenesis as initiation, tumor development and homeostasis of mammary tumors are not only influenced by intrinsic tumor factors but also by extrinsic factors of the tumor microenvironment (Finak, Bertos et al. 2008; Casey, Bond et al. 2009; Trimboli, Cantemir-Stone et al. 2009). Furthermore, the tumor stroma is assumed to support the epithelial tumor cell and even modulate the sensitivity to chemo-radiotherapy (Farmer, Bonnefoi et al. 2009; Tokes, Szasz et al. 2009). An interplay between epithelial tumor cells and tumor associated stromal cells via feedback loop is hypothesized (Martinez-Outschoorn, Pavlides et al. 2011). Beside the deregulation of miRNA expression in tumor cells, a deregulation is also described in cancer associated fibroblasts (Bronisz, Godlewski et al. 2012).

In this study non-neoplastic epithelium and primary tumors were obtained by macrodissection and therefore contained both epithelial and stromal cells. Both cell types may therefore be accountable for the expression of miRNA and snoRNA. The potential differences in the composition of the tissue samples may have contributed to the lack of differences in the expression of most miRNA between primary tumors at different stages of malignancy. The significant up-regulation of miR-21 in non-metastasizing and metastasizing carcinoma compared to normal mammary gland may also be enforced by an expressional increase of both tumor and stromal cells. This conclusion is supported by recent studies on human breast cancer that revealed an overexpression of miR-21 in tumor cells of increased

invasiveness as well as in stromal cells (Rask, Balslev et al. 2011; Niu, Shi et al. 2012). Lymph node metastases on the other hand were obtained by microdissection, which makes the comparison of both tissue samples difficult. Hence, the stromal cells of the macrodissected samples may have contributed to the recorded down-regulation of miRNAs in microdissected metastases when compared to non-neoplastic tissue and primary tumors.

4.5 Therapeutic approaches based on miRNA expression

CMT display the most common cancer afflicting the femal dog (Dorn, Taylor et al. 1968). Similarly, breast cancer is the most frequent tumor in women around the world. Furthermore, its chemoresistance and metastatic behavior makes breast cancer to the second leading cause of cancer related death (Kim, Oh et al. 2011; Ru, Steele et al. 2011). Therefore, apart from biomarkers facilitating early diagnosis and detection of an ideal starting point for intervention novel therapeutic approaches are highly desirable. Advancing elucidation of molecular mechanisms of tumorigenesis may help to develop new therapeutic strategies. Our study reveals a further step towards understanding the role of miRNAs in mammary cancer. In the last years, miRNAs have emerged the focus of cancer therapy. This trend is founded on the observation that miRNA expression is deregulated in cancer compared to non-neoplastic tissue. Furthermore, recent studies suggested that cancer phenotypes can be changed by modulating miRNA expression (Garzon, Marcucci et al. 2010). Two main therapeutic strategies have been established in the recent years. First, miRNA function is inhibited by antisense oligonucleotides. Four classes of antisense oligonucleotides have been developed: locked nucleic acids, anti-miRNA oligonucleotides, antagomirs and miRNA sponges. Second, miRNA function is restored by modulating miRNA expression or substitution of synthetic miRNA mimics. In the ‘miRNA replacement therapy’, the expression of single miRNA species is restored by the use of an adenoviral vector (Kota, Chivukula et al. 2009). In contrast, the ‘miRNAome based strategy’ is aimed at a global restoration of miRNA expression (Melo, Villanueva et al. 2011). The function of particular miRNAs can also be substituted by the use of synthetic oligonucleotides, so called miRNA mimics (Garzon, Marcucci et al. 2010). One outstanding benefit of miRNA based therapy over other therapeutic strategies is the fact that single miRNA species regulate multiple targets and therefore have impact on various pathways involved in tumorigenesis. However, there are only few miRNAs deregulated in cancer cells compared to the extensive alterations in the transcriptome or proteome. Therefore, targeting deregulated cancer related miRNAs might be more efficient and successful than targeting genes or proteins (Garzon, Marcucci et al. 2010). The purpose of any therapy is to maximize its benefit and minimize its off target

effects. The use of tissue specific adenoviral vectors might reduce these off target effects compared to unspecific, systemic drug administration (Michelfelder and Trepel 2009).

Already in 1976, Misdorp and colleagues recommended a double tracked therapy for mammary cancer by treating it both surgically and systemically (Misdorp 1976). The introduction of miRNA based therapy in combination with conventional therapeutic methods could both increase cure rate and improve disease response. Surgical excision of the primary tumor completed with an anti-metastatic miRNA based therapy could be one potential application. The restoration of miR-145, which is down-regulated in metastases compared to all other tissue groups in our study, represents a supposable complementary anti-metastatic therapy. Kim and colleagues found that adenoviral restoration of miR-145 expression leads to suppression of tumor growth and cell motility in breast cancer (Kim, Oh et al. 2011). Beside the direct impact on the tumor, miRNA based therapy can also be aimed at improving the response to treatment or prevention of drug resistance. The development of chemoresistance is one key problem in conventional cancer treatment. Ru et al. could enhance the chemosensitivity of breast cancer cells by inhibition of miR-203 leading to increased SOCS3 expression (Ru, Steele et al. 2011). Our study supported the finding that miR-203 shows a significant expressional increase in neoplastic mammary tissue (adenoma) when compared to non-neoplastic tissue.

Although various promising therapeutic strategies have been successfully developed, miRNA based therapy is still in its infancy. Thus, more studies are needed to verify the role of certain mirRNAs in particular steps of tumorigenesis and to confirm their therapeutic benefit. Furthermore, intrinsic challenges of oligonucleotide base strategies, like bioavailability and cellular uptake, have to be overcome (Garzon, Marcucci et al. 2010; Esteller 2011).

4.6 Conclusion and outlook

This study introduced a canine specific SYBRGreen based real-time PCR assay for the quantification of both miRNA and snoRNA. The malignancy associated expression of 16 miRNAs and 4 snoRNAs was evaluated in non-neoplastic mammary gland and CMT at different stages of malignancy. From all miRNA and snoRNA species tested nine miRNAs and one snoRNA showed a significantly deregulated expression between different tissue types. In detail, the expression of five miRNAs (miR-203, mir-143, miR-21, miR-194, miR-210) was increased in at least one primary tumor group versus non-neoplastic mammary gland. In contrast, one snoRNA (U24) was down-regulated in metastasizing carcinomas when compared to non-neoplastic mammary gland. In the group of lymph node metastases,

the expression of three miRNAs (miR-143, miR-145, miR-101) was decreased when compared to non-neoplastic tissue and all primary tumors. One further miRNA (miR-29b) was down-regulated in lymph node metastases compared to all other groups but non-neoplastic tissue. The expression of only two miRNAs (miR-210, miR-125a) was increased in the metastases. Interestingly, miR-210 showed a continuous up-regulation over the course of malignant progression. In contrast, miR-125a was only up-regulated in metastases when compared to metastasizing carcinomas. Intriguingly, discrimination of primary tumors at different stages of malignancy was only enabled by miR-125a, which showed a down-regulation in metastasizing carcinomas versus adenomas. Thus, miR-125a allows the differentiation between malignant and benign primary tumors and therefore represents a potential predictive biomarker for cancer diagnosis. All miRNA species overexpressed in tumor groups versus non-neoplastic tissue display potential oncogenes. Accordingly, all miRNA species and the single snoRNA species down-regulated in tumor groups versus non-neoplastic tissue might function as tumor suppressors. In future studies, knockdown or overexpression of the miRNA and snoRNA species with different expression levels at different stages of CMT malignancy have to prove their specific role in tumor initiation, development and metastatic behavior.

5 SUMMARY

Malignancy associated expression of microRNA in canine mammary tumors
Marie-Charlotte von Deetzen

Canine mammary tumors are the most common neoplasms in the bitch. Approximately 50 % of the CMT are histologically diagnosed as malignant. Lymphogenous and haematogenous metastatic spread is common and metastatic disease is the most frequent cause of death in these patients. On the basis of their histological features according to the World Health Organization tumor classification, benign adenomas and malignant carcinomas can easily be distinguished in most cases. Despite worldwide research on mammary cancer, molecular details of the complex metastatic cascade remain unclear. Moreover, the question if the metastatic capacity displays an early inherent feature or a property a tumor acquires in the course of malignant progression remains unanswered. Thus the differentiation between metastasizing and non-metastasizing carcinomas before metastasis is clinically detectable still poses an unresolved challenge.

This study investigated the malignancy associated expression of 16 miRNAs and 4 snoRNAs quantitatively in both non-neoplastic mammary gland and mammary tumors at different stages of malignancy. The mammary tumors were diagnosed histologically and grouped into adenomas, non-metastasizing carcinomas, metastasizing carcinomas and lymph node metastases.
The following main hypothesis was tested: Particular miRNA and snoRNA species are specifically expressed in canine mammary tumors at different stages of malignancy. Thus, their expression pattern allows a differentiation between non-neoplastic mammary gland and neoplastic mammary tissue, benign tumors and malignant tumors as well as between non-metastasizing and metastasizing carcinomas. Differentially expressed miRNA and snoRNA represent potential biomarkers for cancer diagnosis.

In total nine miRNAs and one snoRNA showed a significantly altered expression in different tissue types. In detail, the expression of five miRNAs (miR-203, miR-143, miR-21, miR-194, miR-210) was increased in at least one primary tumor group versus non-neoplastic mammary gland. Thus, these up-regulated miRNA species might function as oncogenic oncomirs. In contrast, only one snoRNA (U24) was down-regulated in a primary tumor (metastasizing carcinoma) when compared to non-neoplastic mammary gland and therefore might function as a tumor suppressor. In the group of lymph node metastases, three miRNAs (miR-143, miR-145, miR-101) were down-regulated versus non-neoplastic tissue and all primary tumors. One further miRNA (miR-29b) showed a decreased expression in lymph

node metastases versus all other groups but non-neoplastic tissue. An increased expression in the group of metastases was only identified for two miRNAs (miR-210, miR-125a). Intriguingly, discrimination of primary tumors at different stages of malignancy was only enabled by miR-125a, which showed a down-regulation in metastasizing carcinomas versus adenomas. Thus, miR-125a allows the differentiation between malignant and benign primary mammary tumors and therefore exhibits the potential for a predictive biomarker in cancer diagnosis. However, the study failed to identify biomarkers for metastatic behavior, which would enable the early differentiation between non-metastasizing and metastasizing carcinomas.

In conclusion, the main hypothesis was supported since the expression of some miRNA and snoRNA was specifically deregulated depending on tumor malignancy. The specific oncogenic or tumor suppressive function of the deregulated RNA species has to be validated in further studies. Although the identification of a biomarker for metastatic behavior failed, this study helps to elucidate the molecular carcinogenesis of canine mammary tumors.

6 ZUSAMMENFASSUNG

Malignitäts-abhängige Expression von mikroRNA bei Mammatumoren der Hündin
Marie-Charlotte von Deetzen

Mammatumoren stellen mit einer Prävalenz von 0,2 % die häufigsten Tumoren der Hündin dar. Circa 50 % der kaninen Mammatumoren werden histologisch als bösartig diagnostiziert. Eine lymphogene und hämatogene Streuung ist typisch und Metastasen stellen die häufigste Todesursache für die Patienten dar. Anhand ihrer histologischen Eigenschaften können gutartige Adenoma und bösartige Karzinome gemäß WHO Tumor Klassifizierung in den meisten Fällen gut differenziert werden. Trotz intensiver Forschung auf dem Gebiet der Mammatumoren sind jedoch noch viele Details der komplexen metastatischen Kaskade unklar. So ist bis heute ungeklärt, ob Tumoren bereits sehr früh in ihrer Entwicklung bestimmte Malignitäts-assoziierte Genexpressionsmuster aufweisen oder ob die Fähigkeit zur Metastasierung erst im Verlauf der malignen Progression entsteht. Weiterhin stellt die Prognose zukünftiger bzw. klinisch noch nicht identifizierbarer Metastasierung immer noch eine große Herausforderung dar.

Die vorliegende Studie untersuchte deshalb, ob 16 miRNAs und 4 snoRNA eine Malignitäts-abhängige Expression in histologisch definiertem, nicht-neoplastischem Mammagewebe, Adenomen, nicht metastasierenden Karzinomen, metastasierenden Karzinomen und Lymphknotenmetastasen zeigen.
Die folgende Hypothese wurde getestet: Einzelne miRNA und snoRNA Spezies zeigen eine spezifische Expression in kaninen Mammatumoren unterschiedlicher Dignität. Ihr Expressionsmuster ermöglicht somit die Unterscheidung von nicht-neoplastischem Mammagewebe und Mammatumoren, von gutartigen und bösartigen Mammatumoren sowie von nicht metastasierenden Karzinomen und metastasierenden Karzinomen. Somit stellen diese miRNA und snoRNA Spezies potenzielle Biomarker für die Tumordiagnostik dar.

Neun miRNAs und eine snoRNA zeigten eine signifikant unterschiedliche Expression in verschiedenen Gewebetypen. Im Detail war die Expression von fünf miRNA Spezies (miR-203, miR-143, miR-21, miR-194, miR-210) in mindestens einer Gruppe der Primärtumoren im Vergleich zum nicht-neoplastischen Gewebe erhöht, was auf eine onkogene Funktion hinweisen könnte. Im Gegensatz dazu zeigte nur eine snoRNA (U24) eine verminderte Expression in den Primärtumoren (metastasierende Karzinome) gegenüber dem nicht-neoplastischen Mammagewebe und könnte demnach die Funktion eines Tumorsuppressors aufweisen. In der Gruppe der Lymphknotenmetastasen zeigten drei miRNA Spezies (miR-

143, miR-145, miR-101) eine verminderte Expression im Vergleich zum nicht-neoplastischem Gewebe und allen Primärtumoren. Die Expression einer weiteren miRNA (miR-29b) war in der Gruppe der Metastasen erniedrigt, allerdings nur im Vergleich zu den Primärtumoren. Eine Expressionszunahme in der Gruppe der Metastasen konnte nur für zwei miRNAs (miR-210, miR-125a) festgestellt werden. Die unterschiedliche Malignität der Primärtumoren wurde nur durch das Expressionsmuster einer einzigen RNA-Spezies, miR-125a, reflektiert. Diese miRNA zeigte einen Expressionsrückgang in der Gruppe der metastasierenden Karzinome im Vergleich zu den Adenomen. Damit ermöglicht miR-125a eine Unterscheidung von gutartigen und bösartigen Mammatumoren und hat somit das Potential eines prognostischen Biomarkers für die Tumordiagnostik. Ein Biomarker zur Differenzierung metastasierender und nicht-metastasierender Tumoren konnte in dieser Studie nicht identifiziert werden.

Abschließend kann die Hypothese soweit unterstützt werden, dass zumindest einzelne miRNA und snoRNA Spezies abhängig von der Tumor-Dignität spezifisch dereguliert sind. Die Identifizierung eines miRNA- oder snoRNA-basierten Biomarkers für metastatisches Verhalten von kanine Mammatumoren war jedoch nicht möglich. Dennoch gibt diese Studie einen weiteren Einblick in die molekulare Karzinogenese kaniner Mammatumoren.

7 REFERENCES

Alenza, D. P.; Rutteman, G. R.; Pena, L.; Beynen, A. C.; Cuesta, P. (1998):
Relation between habitual diet and canine mammary tumors in a case-control study.
Journal of Veterinary Internal Medicine. 12(3), 132-139.
URL:<Go to ISI>://WOS:000073680700002.

Allred, D. C.; Mohsin, S. K.; Fuqua, S. A. (2001):
Histological and biological evolution of human premalignant breast disease.
Endocr Relat Cancer. 8(1), 47-61.
URL:http://www.ncbi.nlm.nih.gov/pubmed/11350726.

Arpino, G.; Laucirica, R.; Elledge, R. M. (2005):
Premalignant and in situ breast disease: biology and clinical implications.
Ann Intern Med. 143(6), 446-57.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16172443.

Bachellerie, J. P.; Cavaille, J. (1997):
Guiding ribose methylation of rRNA.
Trends Biochem Sci. 22(7), 257-61.
URL:http://www.ncbi.nlm.nih.gov/pubmed/9255067.

Bachellerie, J. P.; Cavaille, J.; Huttenhofer, A. (2002):
The expanding snoRNA world.
Biochimie. 84(8), 775-790.
URL:<Go to ISI>://WOS:000179472600011.

Balakin, A. G.; Smith, L.; Fournier, M. J. (1996):
The RNA world of the nucleolus: two major families of small RNAs defined by different box elements with related functions.
Cell. 86(5), 823-34.
URL:http://www.ncbi.nlm.nih.gov/pubmed/8797828.

Barone, R. (1990):
Mamelles. -
Paris: Éditions vigot. -.
(Splanchnologie II.4.).

Barrett, J. C. (1993):
Mechanisms of multistep carcinogenesis and carcinogen risk assessment.
Environ Health Perspect. 100, 9-20.
URL:http://www.ncbi.nlm.nih.gov/pubmed/8354184.

Bartel, D. P. (2009):
MicroRNAs: target recognition and regulatory functions.
Cell. 136(2), 215-33.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19167326.

Beauvais, W.; Cardwell, J. M.; Brodbelt, D. C. (2012):
The effect of neutering on the risk of mammary tumours in dogs--a systematic review.
J Small Anim Pract. 53(6), 314-22.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22647210.

Bednarz-Knoll, N.; Alix-Panabieres, C.; Pantel, K. (2011):
Clinical relevance and biology of circulating tumor cells.
Breast Cancer Research. 13(6).
URL:<Go to ISI>://WOS:000301173700034.

Benes, V.; Castoldi, M. (2010):
Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available.
Methods. 50(4), 244-9.
URL:http://www.ncbi.nlm.nih.gov/pubmed/20109550.

Benjamin, S. A.; Lee, A. C.; Saunders, W. J. (1999):
Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles.
Vet Pathol. 36(5), 423-36.
URL:http://www.ncbi.nlm.nih.gov/pubmed/10490210.

Boggs, R. M.; Wright, Z. M.; Stickney, M. J.; Porter, W. W.; Murphy, K. E. (2008):
MicroRNA expression in canine mammary cancer.
Mamm Genome. 19(7-8), 561-9.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18665421.

Borge, K. S.; Borresen-Dale, A. L.; Lingaas, F. (2011):
Identification of genetic variation in 11 candidate genes of canine mammary tumour.
Vet Comp Oncol. 9(4), 241-50.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22077404.

Borre, M.; Offersen, B. V.; Nerstrom, B.; Overgaard, J. (1998):
Microvessel density predicts survival in prostate cancer patients subjected to watchful waiting.
Br J Cancer. 78(7), 940-4.
URL:http://www.ncbi.nlm.nih.gov/pubmed/9764587.

Brattelid, T.; Aarnes, E. K.; Helgeland, E.; Guvaag, S.; Eichele, H.; Jonassen, A. K. (2011):
Normalization strategy is critical for the outcome of miRNA expression analyses in the rat heart.
Physiol Genomics. 43(10), 604-10.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21177382.

Brodey, R. S.; Fidler, I. J.; Howson, A. E. (1966):
The relationship of estrus cycle irregularity, pseudo-pregancy, and pregnancy of the development of canine mammary neoplasm.
J Am Vet Med Assoc. 149, 1047-1049.

Bronisz, A.; Godlewski, J.; Wallace, J. A.; Merchant, A. S.; Nowicki, M. O.; Mathsyaraja, H.; Srinivasan, R.; Trimboli, A. J.; Martin, C. K.; Li, F.; Yu, L.; Fernandez, S. A.; Pecot, T.; Rosol, T. J.; Cory, S.; Hallett, M.; Park, M.; Piper, M. G.; Marsh, C. B.; Yee, L. D.; Jimenez, R. E.; Nuovo, G.; Lawler, S. E.; Chiocca, E. A.; Leone, G.; Ostrowski, M. C. (2012):
Reprogramming of the tumour microenvironment by stromal PTEN-regulated miR-320.
Nat Cell Biol. 14(2), 159-67.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22179046.

Calin, G. A.; Dumitru, C. D.; Shimizu, M.; Bichi, R.; Zupo, S.; Noch, E.; Aldler, H.; Rattan, S.; Keating, M.; Rai, K.; Rassenti, L.; Kipps, T.; Negrini, M.; Bullrich, F.; Croce, C. M. (2002):
Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia.
Proc Natl Acad Sci U S A. 99(24), 15524-9.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12434020.

Calin, G. A.; Sevignani, C.; Dumitru, C. D.; Hyslop, T.; Noch, E.; Yendamuri, S.; Shimizu, M.; Rattan, S.; Bullrich, F.; Negrini, M.; Croce, C. M. (2004):
Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers.
Proc Natl Acad Sci U S A. 101(9), 2999-3004.
URL:http://www.ncbi.nlm.nih.gov/pubmed/14973191.

Callahan, R.; Campbell, G. (1989):
Mutations in human breast cancer: an overview.
J Natl Cancer Inst. 81(23), 1780-6.
URL:http://www.ncbi.nlm.nih.gov/pubmed/2685334.

Carlsson, J.; Helenius, G.; Karlsson, M.; Lubovac, Z.; Andren, O.; Olsson, B.; Klinga-Levan, K. (2010):
Validation of suitable endogenous control genes for expression studies of miRNA in prostate cancer tissues.
Cancer Genet Cytogenet. 202(2), 71-5.
URL:http://www.ncbi.nlm.nih.gov/pubmed/20875868.

Casey, T.; Bond, J.; Tighe, S.; Hunter, T.; Lintault, L.; Patel, O.; Eneman, J.; Crocker, A.; White, J.; Tessitore, J.; Stanley, M.; Harlow, S.; Weaver, D.; Muss, H.; Plaut, K. (2009):
Molecular signatures suggest a major role for stromal cells in development of invasive breast cancer.
Breast Cancer Res Treat. 114(1), 47-62.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18373191.

Chambers, A. F.; Groom, A. C.; MacDonald, I. C. (2002):
Dissemination and growth of cancer cells in metastatic sites.
Nat Rev Cancer. 2(8), 563-72.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12154349.

Chang, C. C.; Tsai, M. H.; Liao, J. W.; Chan, J. P.; Wong, M. L.; Chang, S. C. (2009):
Evaluation of hormone receptor expression for use in predicting survival of female dogs with malignant mammary gland tumors.
J Am Vet Med Assoc. 235(4), 391-6.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19681719.

Chang, K. H.; Mestdagh, P.; Vandesompele, J.; Kerin, M. J.; Miller, N. (2010):
MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer.
BMC Cancer. 10, 173.
URL:http://www.ncbi.nlm.nih.gov/pubmed/20429937.

Chang, L. S.; Lin, S. Y.; Lieu, A. S.; Wu, T. L. (2002):
Differential expression of human 5S snoRNA genes.
Biochem Biophys Res Commun. 299(2), 196-200.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12437969.

Chang, S. C.; Chang, C. C.; Chang, T. J.; Wong, M. L. (2005):
Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998-2002).
J Am Vet Med Assoc. 227(10), 1625-9.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16313041.

Chen, C.; Ridzon, D. A.; Broomer, A. J.; Zhou, Z.; Lee, D. H.; Nguyen, J. T.; Barbisin, M.; Xu, N. L.; Mahuvakar, V. R.; Andersen, M. R.; Lao, K. Q.; Livak, K. J.; Guegler, K. J. (2005):
Real-time quantification of microRNAs by stem-loop RT-PCR.
Nucleic Acids Res. 33(20), e179.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16314309.

Cheng, A. M.; Byrom, M. W.; Shelton, J.; Ford, L. P. (2005):
Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis.
Nucleic Acids Res. 33(4), 1290-7.
URL:http://www.ncbi.nlm.nih.gov/pubmed/15741182.

Cissell, K. A.; Campbell, S.; Deo, S. K. (2008):
Rapid, single-step nucleic acid detection.
Anal Bioanal Chem. 391(7), 2577-81.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18563395.

Cissell, K. A.; Deo, S. K. (2009):
Trends in microRNA detection.
Anal Bioanal Chem. 394(4), 1109-16.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19367400.

Cissell, K. A.; Rahimi, Y.; Shrestha, S.; Hunt, E. A.; Deo, S. K. (2008):
Bioluminescence-based detection of microRNA, miR21 in breast cancer cells.
Anal Chem. 80(7), 2319-25.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18302417.

Cleary, M. P.; Grossmann, M. E. (2009):
Minireview: Obesity and breast cancer: the estrogen connection.
Endocrinology. 150(6), 2537-42.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19372199.

Cleary, M. P.; Grossmann, M. E.; Ray, A. (2010):
Effect of obesity on breast cancer development.
Vet Pathol. 47(2), 202-13.
URL:http://www.ncbi.nlm.nih.gov/pubmed/20124008.

Croce, C. M. (2008):
Oncogenes and cancer.
N Engl J Med. 358(5), 502-11.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18234754.

Cui, H.; Grosso, S.; Schelter, F.; Mari, B.; Kruger, A. (2012):
On the Pro-Metastatic Stress Response to Cancer Therapies: Evidence for a Positive Co-Operation between TIMP-1, HIF-1alpha, and miR-210.
Front Pharmacol. 3, 134.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22807917.

Czech, B.; Hannon, G. J. (2011):
Small RNA sorting: matchmaking for Argonautes.
Nat Rev Genet. 12(1), 19-31.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21116305.

Davoren, P. A.; McNeill, R. E.; Lowery, A. J.; Kerin, M. J.; Miller, N. (2008):
Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer.
BMC Mol Biol. 9, 76.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18718003.

de Jong, J. S.; van Diest, P. J.; Baak, J. P. (2000):
Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer.
Histopathology. 36(4), 306-12.
URL:http://www.ncbi.nlm.nih.gov/pubmed/10759944.

Dong, X. Y.; Guo, P.; Boyd, J.; Sun, X.; Li, Q.; Zhou, W.; Dong, J. T. (2009):
Implication of snoRNA U50 in human breast cancer.
J Genet Genomics. 36(8), 447-54.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19683667.

Dong, X. Y.; Rodriguez, C.; Guo, P.; Sun, X.; Talbot, J. T.; Zhou, W.; Petros, J.; Li, Q.; Vessella, R. L.; Kibel, A. S.; Stevens, V. L.; Calle, E. E.; Dong, J. T. (2008):
SnoRNA U50 is a candidate tumor-suppressor gene at 6q14.3 with a mutation associated with clinically significant prostate cancer.
Human Molecular Genetics. 17(7), 1031-42.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18202102.

Dorn, C. R.; Taylor, D. O.; Schneider, R.; Hibbard, H. H.; Klauber, M. R. (1968):
Survey of animal neoplasms in Alameda and Contra Costa Counties, California. II. Cancer morbidity in dogs and cats from Alameda County.
J Natl Cancer Inst. 40(2), 307-18.
URL:http://www.ncbi.nlm.nih.gov/pubmed/5694272.

Egenvall, A.; Bonnett, B. N.; Ohagen, P.; Olson, P.; Hedhammar, A.; von Euler, H. (2005):
Incidence of and survival after mammary tumors in a population of over 80,000 insured female dogs in Sweden from 1995 to 2002.
Prev Vet Med. 69(1-2), 109-27.
URL:http://www.ncbi.nlm.nih.gov/pubmed/15899300.

Else, R. W.; Hannant, D. (1979):
Some Epidemiological Aspects of Mammary Neoplasia in the Bitch.
Veterinary Record. 104(14), 296-304.
URL:<Go to ISI>://WOS:A1979GR05700002.

Elston, C. W.; Ellis, I. O. (1991):
Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up.
Histopathology. 19(5), 403-10.
URL:http://www.ncbi.nlm.nih.gov/pubmed/1757079.

Esquela-Kerscher, A.; Slack, F. J. (2006):
Oncomirs - microRNAs with a role in cancer.
Nat Rev Cancer. 6(4), 259-69.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16557279.

Esteller, M. (2011):
Non-coding RNAs in human disease.
Nat Rev Genet. 12(12), 861-74.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22094949.

Estrela-Lima, A.; Araujo, M. S.; Costa-Neto, J. M.; Teixeira-Carvalho, A.; Barrouin-Melo, S. M.; Cardoso, S. V.; Martins-Filho, O. A.; Serakides, R.; Cassali, G. D. (2010):
Immunophenotypic features of tumor infiltrating lymphocytes from mammary carcinomas in female dogs associated with prognostic factors and survival rates.
BMC Cancer. 10, 256.
URL:http://www.ncbi.nlm.nih.gov/pubmed/20525350.

Farazi, T. A.; Spitzer, J. I.; Morozov, P.; Tuschl, T. (2011):
miRNAs in human cancer.
J Pathol. 223(2), 102-15.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21125669.

Farmer, P.; Bonnefoi, H.; Anderle, P.; Cameron, D.; Wirapati, P.; Becette, V.; Andre, S.; Piccart, M.; Campone, M.; Brain, E.; Macgrogan, G.; Petit, T.; Jassem, J.; Bibeau, F.; Blot, E.; Bogaerts, J.; Aguet, M.; Bergh, J.; Iggo, R.; Delorenzi, M. (2009):
A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer.
Nat Med. 15(1), 68-74.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19122658.

Fasanaro, P.; D'Alessandra, Y.; Di Stefano, V.; Melchionna, R.; Romani, S.; Pompilio, G.; Capogrossi, M. C.; Martelli, F. (2008):
MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3.
Journal of Biological Chemistry. 283(23), 15878-15883.
URL:<Go to ISI>://WOS:000256332500046.

Ferreira, E.; Bertagnolli, A. C.; Cavalcanti, M. F.; Schmitt, F. C.; Cassali, G. D. (2009):
The relationship between tumour size and expression of prognostic markers in benign and malignant canine mammary tumours.
Veterinary and Comparative Oncology. 7(4), 230-235.
URL:<Go to ISI>://WOS:000271517000003.

Finak, G.; Bertos, N.; Pepin, F.; Sadekova, S.; Souleimanova, M.; Zhao, H.; Chen, H.; Omeroglu, G.; Meterissian, S.; Omeroglu, A.; Hallett, M.; Park, M. (2008):
Stromal gene expression predicts clinical outcome in breast cancer.
Nat Med. 14(5), 518-27.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18438415.

Folkman, J. (1990):
What is the evidence that tumors are angiogenesis dependent?
J Natl Cancer Inst. 82(1), 4-6.
URL:http://www.ncbi.nlm.nih.gov/pubmed/1688381.

Friedman, L. M.; Avraham, K. B. (2009):
MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness.
Mammalian Genome. 20(9-10), 581-603.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19876605.

Garzon, R.; Marcucci, G.; Croce, C. M. (2010):
Targeting microRNAs in cancer: rationale, strategies and challenges.
Nat Rev Drug Discov. 9(10), 775-89.
URL:http://www.ncbi.nlm.nih.gov/pubmed/20885409.

Gee, H. E.; Buffa, F. M.; Camps, C.; Ramachandran, A.; Leek, R.; Taylor, M.; Patil, M.; Sheldon, H.; Betts, G.; Homer, J.; West, C.; Ragoussis, J.; Harris, A. L. (2011):
The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis.
British Journal of Cancer. 104(7), 1168-1177.
URL:<Go to ISI>://WOS:000288938100015.

Gilbertson, R. J.; Graham, T. A. (2012):
Cancer: Resolving the stem-cell debate.
Nature. 488(7412), 462-3.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22919708.

Giles, R. C.; Kwapien, R. P.; Geil, R. G.; Casey, H. W. (1978):
Mammary nodules in beagle dogs administered investigational oral contraceptive steroids.
J Natl Cancer Inst. 60(6), 1351-64.
URL:http://www.ncbi.nlm.nih.gov/pubmed/650701.

Giraldez, A.:
from http://www.yale.edu/giraldezlab/Research.html.

Goldschmidt, M.; Pena, L.; Rasotto, R.; Zappulli, V. (2011):
Classification and grading of canine mammary tumors.
Vet Pathol. 48(1), 117-31.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21266722.

Graham, J. C.; Myers, R. K. (1999):
The prognostic significance of angiogenesis in canine mammary tumors.
J Vet Intern Med. 13(5), 416-8.
URL:http://www.ncbi.nlm.nih.gov/pubmed/10499723.

Greaves, M.; Maley, C. C. (2012):
Clonal evolution in cancer.
Nature. 481(7381), 306-13.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22258609.

Grosshans, H.; Filipowicz, W. (2008):
Molecular biology: the expanding world of small RNAs.
Nature. 451(7177), 414-6.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18216846.

Guo, X.; Wu, Y.; Hartley, R. S. (2009):
MicroRNA-125a represses cell growth by targeting HuR in breast cancer.
RNA Biol. 6(5), 575-83.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19875930.

Hampe, J. F.; Misdorp, W. (1974):
Tumours and dysplasias of the mammary gland.
Bull World Health Organ. 50(1-2), 111-33.
URL:http://www.ncbi.nlm.nih.gov/pubmed/4371737.

Hartmann, L. C.; Sellers, T. A.; Frost, M. H.; Lingle, W. L.; Degnim, A. C.; Ghosh, K.; Vierkant, R. A.; Maloney, S. D.; Pankratz, V. S.; Hillman, D. W.; Suman, V. J.; Johnson, J.; Blake, C.; Tlsty, T.; Vachon, C. M.; Melton, L. J., 3rd; Visscher, D. W. (2005):
Benign breast disease and the risk of breast cancer.
N Engl J Med. 353(3), 229-37.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16034008.

Hellmen, E.; Bergstrom, R.; Holmberg, L.; Spangberg, I. B.; Hansson, K.; Lindgren, A. (1993):
Prognostic factors in canine mammary tumors: a multivariate study of 202 consecutive cases.
Vet Pathol. 30(1), 20-7.
URL:http://www.ncbi.nlm.nih.gov/pubmed/8442324.

Hon, L. S.; Zhang, Z. (2007):
The roles of binding site arrangement and combinatorial targeting in microRNA repression of gene expression.
Genome Biol. 8(8), R166.
URL:http://www.ncbi.nlm.nih.gov/pubmed/17697356.

Iizuka, D.; Imaoka, T.; Nishimura, M.; Kawai, H.; Suzuki, F.; Shimada, Y. (2013):
Aberrant microRNA expression in radiation-induced rat mammary cancer: the potential role of miR-194 overexpression in cancer cell proliferation.
Radiat Res. 179(2), 151-9.
URL:http://www.ncbi.nlm.nih.gov/pubmed/23273170.

Iorio, M. V.; Ferracin, M.; Liu, C. G.; Veronese, A.; Spizzo, R.; Sabbioni, S.; Magri, E.; Pedriali, M.; Fabbri, M.; Campiglio, M.; Menard, S.; Palazzo, J. P.; Rosenberg, A.; Musiani, P.; Volinia, S.; Nenci, I.; Calin, G. A.; Querzoli, P.; Negrini, M.; Croce, C. M. (2005):
MicroRNA gene expression deregulation in human breast cancer.
Cancer Res. 65(16), 7065-70.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16103053.

Jiang, W. G.; Martin, T. A.; Mansel, R. E. (2002):
Molecular detection of micro-metastasis in breast cancer.
Crit Rev Oncol Hematol. 43(1), 13-31.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12098605.

Karayannopoulou, M.; Kaldrymidou, E.; Constantinidis, T. C.; Dessiris, A. (2005):
Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method.
J Comp Pathol. 133(4), 246-52.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16202421.

Kim, J.; Inoue, K.; Ishii, J.; Vanti, W. B.; Voronov, S. V.; Murchison, E.; Hannon, G.; Abeliovich, A. (2007):
A MicroRNA feedback circuit in midbrain dopamine neurons.
Science. 317(5842), 1220-4.
URL:http://www.ncbi.nlm.nih.gov/pubmed/17761882.

Kim, S. J.; Oh, J. S.; Shin, J. Y.; Lee, K. D.; Sung, K. W.; Nam, S. J.; Chun, K. H. (2011):
Development of microRNA-145 for therapeutic application in breast cancer.
J Control Release. 155(3), 427-34.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21723890.

Kiss, A. M.; Jady, B. E.; Bertrand, E.; Kiss, T. (2004):
Human box H/ACA pseudouridylation guide RNA machinery.
Molecular and Cellular Biology. 24(13), 5797-807.
URL:http://www.ncbi.nlm.nih.gov/pubmed/15199136.

Kiss, T. (2002):
Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions.
Cell. 109(2), 145-8.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12007400.

Klopfleisch, R.; von Euler, H.; Sarli, G.; Pinho, S. S.; Gartner, F.; Gruber, A. D. (2011):
Molecular carcinogenesis of canine mammary tumors: news from an old disease.
Vet Pathol. 48(1), 98-116.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21149845.

Kota, J.; Chivukula, R. R.; O'Donnell, K. A.; Wentzel, E. A.; Montgomery, C. L.; Hwang, H. W.; Chang, T. C.; Vivekanandan, P.; Torbenson, M.; Clark, K. R.; Mendell, J. R.; Mendell, J. T. (2009):
Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model.
Cell. 137(6), 1005-17.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19524505.

Kulshreshtha, R.; Ferracin, M.; Wojcik, S. E.; Garzon, R.; Alder, H.; Agosto-Perez, F. J.; Davuluri, R.; Liu, C. G.; Croce, C. M.; Negrini, M.; Calin, G. A.; Ivan, M. (2007):
A microRNA signature of hypoxia.
Molecular and Cellular Biology. 27(5), 1859-1867.
URL:<Go to ISI>://WOS:000244305500025.

Kurzman, I. D.; Gilbertson, S. R. (1986):
Prognostic factors in canine mammary tumors.
Semin Vet Med Surg (Small Anim). 1(1), 25-32.
URL:http://www.ncbi.nlm.nih.gov/pubmed/3507784.

Kvale, G. (1992):
Reproductive factors in breast cancer epidemiology.
Acta Oncol. 31(2), 187-94.
URL:http://www.ncbi.nlm.nih.gov/pubmed/1622633.

Le, M. T.; Teh, C.; Shyh-Chang, N.; Xie, H.; Zhou, B.; Korzh, V.; Lodish, H. F.; Lim, B. (2009):
MicroRNA-125b is a novel negative regulator of p53.
Genes Dev. 23(7), 862-76.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19293287.

Lebeau, A. (1953):
L'age du chien et celui de l'homme.
Bulletin de l'Academie Vétérinaire de France. 26, 229-32.

Lee, Y.; Kim, M.; Han, J.; Yeom, K. H.; Lee, S.; Baek, S. H.; Kim, V. N. (2004):
MicroRNA genes are transcribed by RNA polymerase II.
EMBO J. 23(20), 4051-60.
URL:http://www.ncbi.nlm.nih.gov/pubmed/15372072.

Li, W.; Ruan, K. (2009):
MicroRNA detection by microarray.
Anal Bioanal Chem. 394(4), 1117-24.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19132354.

Lujambio, A.; Lowe, S. W. (2012):
The microcosmos of cancer.
Nature. 482(7385), 347-55.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22337054.

MacEwen, E. G.; Patnaik, A. K.; Harvey, H. J.; Panko, W. B. (1982):
Estrogen receptors in canine mammary tumors.
Cancer Res. 42(6), 2255-9.
URL:http://www.ncbi.nlm.nih.gov/pubmed/7074608.

MacEwen, E. W., S. (1996):
Tumors of the mammary gland. Small Animal Oncology. -
Philadelphia: Saunders Company. -.).

Marconato, L.; Romanelli, G.; Stefanello, D.; Giacoboni, C.; Bonfanti, U.; Bettini, G.; Finotello, R.; Verganti, S.; Valenti, P.; Ciaramella, L.; Zini, E. (2009):
Prognostic factors for dogs with mammary inflammatory carcinoma: 43 cases (2003-2008).
J Am Vet Med Assoc. 235(8), 967-72.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19827983.

Martinez-Outschoorn, U. E.; Pavlides, S.; Howell, A.; Pestell, R. G.; Tanowitz, H. B.; Sotgia, F.; Lisanti, M. P. (2011):
Stromal-epithelial metabolic coupling in cancer: integrating autophagy and metabolism in the tumor microenvironment.
Int J Biochem Cell Biol. 43(7), 1045-51.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21300172.

Mattick, J. S.; Makunin, I. V. (2005):
Small regulatory RNAs in mammals.
Human Molecular Genetics. 14, R121-R132.
URL:<Go to ISI>://WOS:000228241000016.

Melo, S.; Villanueva, A.; Moutinho, C.; Davalos, V.; Spizzo, R.; Ivan, C.; Rossi, S.; Setien, F.; Casanovas, O.; Simo-Riudalbas, L.; Carmona, J.; Carrere, J.; Vidal, A.; Aytes, A.; Puertas, S.; Ropero, S.; Kalluri, R.; Croce, C. M.; Calin, G. A.; Esteller, M. (2011):
Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing.
Proc Natl Acad Sci U S A. 108(11), 4394-9.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21368194.

Mendell, J. T. (2005):
MicroRNAs: critical regulators of development, cellular physiology and malignancy.
Cell Cycle. 4(9), 1179-84.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16096373.

Michelfelder, S.; Trepel, M. (2009):
Adeno-associated viral vectors and their redirection to cell-type specific receptors.
Adv Genet. 67, 29-60.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19914449.

Millanta, F.; Silvestri, G.; Vaselli, C.; Citi, S.; Pisani, G.; Lorenzi, D.; Poli, A. (2006):
The role of vascular endothelial growth factor and its receptor Flk-1/KDR in promoting tumour angiogenesis in feline and canine mammary carcinomas: a preliminary study of autocrine and paracrine loops.
Res Vet Sci. 81(3), 350-7.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16556453.

Miller, M. E.; Christensen, G. C.; Evand, H. E. (1964):
The mammary gland. Anatomy of the dog. -
Philadelphia: W.B. Saunders. -.).

Misdorp, W. (2002):
Tumors of the mammary gland.
In: Tumors in Domestic Animals. / Hrsg. D. J. Meuten. - Ames: Iowa State Press. -. S. 575-606.). -.

Misdorp, W.; Else, R. W.; Hellmen, E.; Lipscomb, T. P. (1999):
Histological Classification of Mammary Tumors of the Dog and the Cat. 2nd edn. -
Washington D.C.: Armed Forces Institute of Pathology. -.).

Misdorp, W., Hart AA. (1976):
Canine mammary cancer II. Therapy and causes of death.
British Small Animal Veterinary Association.

Mocellin, S.; Pasquali, S.; Pilati, P. (2009):
Oncomirs: from tumor biology to molecularly targeted anticancer strategies.
Mini Rev Med Chem. 9(1), 70-80.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19149661.

Moe, L. (2001):
Population-based incidence of mammary tumours in some dog breeds.
J Reprod Fertil Suppl. 57, 439-43.
URL:http://www.ncbi.nlm.nih.gov/pubmed/11787188.

Mol, J. A.; van Garderen, E.; Rutteman, G. R.; Rijnberk, A. (1996):
New insights in the molecular mechanism of progestin-induced proliferation of mammary epithelium: induction of the local biosynthesis of growth hormone (GH) in the mammary glands of dogs, cats and humans.
J Steroid Biochem Mol Biol. 57(1-2), 67-71.
URL:http://www.ncbi.nlm.nih.gov/pubmed/8645618.

Mortarino, M.; Gioia, G.; Gelain, M. E.; Albonico, F.; Roccabianca, P.; Ferri, E.; Comazzi, S. (2010):
Identification of suitable endogenous controls and differentially expressed microRNAs in canine fresh-frozen and FFPE lymphoma samples.
Leuk Res. 34(8), 1070-7.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19945163.

Mottolese, M.; Morelli, L.; Agrimi, U.; Benevolo, M.; Sciarretta, F.; Antonucci, G.; Natali, P. G. (1994):
Spontaneous canine mammary tumors. A model for monoclonal antibody diagnosis and treatment of human breast cancer.
Lab Invest. 71(2), 182-7.
URL:http://www.ncbi.nlm.nih.gov/pubmed/8078297.

Moulton, J. E. (1990):
Tumors of the mammary gland.
In: Tumors in domestic animals. / Hrsg. M. J.E. - Berkeley, CA: University of California Press. -. S. 518-552.3rd ed.). -.

Mourtada-Maarabouni, M.; Pickard, M. R.; Hedge, V. L.; Farzaneh, F.; Williams, G. T. (2009):
GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer.
Oncogene. 28(2), 195-208.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18836484.

Mouser, P.; Miller, M. A.; Antuofermo, E.; Badve, S. S.; Mohammed, S. I. (2010):
Prevalence and classification of spontaneous mammary intraepithelial lesions in dogs without clinical mammary disease.
Vet Pathol. 47(2), 275-84.
URL:http://www.ncbi.nlm.nih.gov/pubmed/20106771.

Murakami, Y.; Tateyama, S.; Rungsipipat, A.; Uchida, K.; Yamaguchi, R. (2000):
Immunohistochemical analysis of cyclin A, cyclin D1 and P53 in mammary tumors, squamous cell carcinomas and basal cell tumors of dogs and cats.
J Vet Med Sci. 62(7), 743-50.
URL:http://www.ncbi.nlm.nih.gov/pubmed/10945293.

Neely, L. A.; Patel, S.; Garver, J.; Gallo, M.; Hackett, M.; McLaughlin, S.; Nadel, M.; Harris, J.; Gullans, S.; Rooke, J. (2006):
A single-molecule method for the quantitation of microRNA gene expression.
Nat Methods. 3(1), 41-6.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16369552.

Niu, J.; Shi, Y.; Tan, G.; Yang, C. H.; Fan, M.; Pfeffer, L. M.; Wu, Z. H. (2012):
DNA damage induces NF-kappaB-dependent microRNA-21 up-regulation and promotes breast cancer cell invasion.
J Biol Chem. 287(26), 21783-95.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22547075.

Orom, U. A.; Nielsen, F. C.; Lund, A. H. (2008):
MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation.
Mol Cell. 30(4), 460-71.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18498749.

Otoni, C. C.; Rahal, S. C.; Vulcano, L. C.; Ribeiro, S. M.; Hette, K.; Giordano, T.; Doiche, D. P.; Amorim, R. L. (2010):
Survey radiography and computerized tomography imaging of the thorax in female dogs with mammary tumors.
Acta Vet Scand. 52, 20.
URL:http://www.ncbi.nlm.nih.gov/pubmed/20214816.

Owen, L. N. (1980):
TNM Classification of Tumors in Domestic Animals. -
Geneva: World Health Organization. -.
(World Health Organization VPH/CMO/80.20.).

Parodi, A. L.; Misdorp, W.; Mialot, J. P.; Mialot, M.; Hart, A. A.; Hurtrel, M.; Salomon, J. C. (1983):
Intratumoral BCG and Corynebacterium parvum therapy of canine mammary tumours before radical mastectomy.
Cancer Immunol Immunother. 15(3), 172-7.
URL:http://www.ncbi.nlm.nih.gov/pubmed/6555059.

Patsikas, M. N.; Dessiris, A. (1996a):
The lymph drainage of the mammary glands in the bitch: a lymphographic study. Part I: The 1st, 2nd, 4th and 5th mammary glands.
Anat Histol Embryol. 25(2), 131-8.
URL:http://www.ncbi.nlm.nih.gov/pubmed/8766408.

Patsikas, M. N.; Dessiris, A. (1996b):
The lymph drainage of the mammary glands in the Bitch: a lymphographic study. Part II: The 3rd mammary gland.
Anat Histol Embryol. 25(2), 139-43.
URL:http://www.ncbi.nlm.nih.gov/pubmed/8766409.

Patsikas, M. N.; Karayannopoulou, M.; Kaldrymidoy, E.; Papazoglou, L. G.; Papadopoulou, P. L.; Tzegas, S. I.; Tziris, N. E.; Kaitzis, D. G.; Dimitriadis, A. S.; Dessiris, A. K. (2006):
The lymph drainage of the neoplastic mammary glands in the bitch: a lymphographic study.
Anat Histol Embryol. 35(4), 228-34.
URL:http://www.ncbi.nlm.nih.gov/pubmed/16836586.

Peltier, H. J.; Latham, G. J. (2008):
Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues.
RNA. 14(5), 844-52.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18375788.

Pereira, C. T.; Rahal, S. C.; de Carvalho Balieiro, J. C.; Ribeiro, A. A. (2003):
Lymphatic drainage on healthy and neoplasic mammary glands in female dogs: can it really be altered?
Anat Histol Embryol. 32(5), 282-90.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12969028.

Perez Alenza, M. D.; Pena, L.; Nieto, A. I.; Castano, M. (1997):
Clinical and pathological prognostic factors in canine mammary tumors.
Ann Ist Super Sanita. 33(4), 581-5.
URL:http://www.ncbi.nlm.nih.gov/pubmed/9616968.

Philibert, J. C.; Snyder, P. W.; Glickman, N.; Glickman, L. T.; Knapp, D. W.; Waters, D. J. (2003):
Influence of host factors on survival in dogs with malignant mammary gland tumors.
J Vet Intern Med. 17(1), 102-6.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12564734.

Queiroga, F. L.; Perez-Alenza, M. D.; Silvan, G.; Pena, L.; Lopes, C.; Illera, J. C. (2005):
Role of steroid hormones and prolactin in canine mammary cancer.
J Steroid Biochem Mol Biol. 94(1-3), 181-7.
URL:http://www.ncbi.nlm.nih.gov/pubmed/15862964.

Rask, L.; Balslev, E.; Jorgensen, S.; Eriksen, J.; Flyger, H.; Moller, S.; Hogdall, E.; Litman, T.; Nielsen, B. S. (2011):
High expression of miR-21 in tumor stroma correlates with increased cancer cell proliferation in human breast cancer.
APMIS. 119(10), 663-73.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21917003.

Restucci, B.; De Vico, G.; Maiolino, P. (2000):
Evaluation of angiogenesis in canine mammary tumors by quantitative platelet endothelial cell adhesion molecule immunohistochemistry.
Vet Pathol. 37(4), 297-301.
URL:http://www.ncbi.nlm.nih.gov/pubmed/10896390.

Rossi, S.; Sevignani, C.; Nnadi, S. C.; Siracusa, L. D.; Calin, G. A. (2008):
Cancer-associated genomic regions (CAGRs) and noncoding RNAs: bioinformatics and therapeutic implications.
Mamm Genome. 19(7-8), 526-40.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18636290.

Rottiers, V.; Najafi-Shoushtari, S. H.; Kristo, F.; Gurumurthy, S.; Zhong, L.; Li, Y.; Cohen, D. E.; Gerszten, R. E.; Bardeesy, N.; Mostoslavsky, R.; Naar, A. M. (2011):
MicroRNAs in metabolism and metabolic diseases.
Cold Spring Harb Symp Quant Biol. 76, 225-33.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22156303.

Ru, P.; Steele, R.; Hsueh, E. C.; Ray, R. B. (2011):
Anti-miR-203 Upregulates SOCS3 Expression in Breast Cancer Cells and Enhances Cisplatin Chemosensitivity.
Genes Cancer. 2(7), 720-7.
URL:http://www.ncbi.nlm.nih.gov/pubmed/22207897.

Rutteman, G. R. (1990):
Hormones and mammary tumour disease in the female dog: an update.
In Vivo. 4(1), 33-40.
URL:http://www.ncbi.nlm.nih.gov/pubmed/2103839.

Rutteman, G. R.; Misdorp, W.; Blankenstein, M. A.; van den Brom, W. E. (1988):
Oestrogen (ER) and progestin receptors (PR) in mammary tissue of the female dog: different receptor profile in non-malignant and malignant states.
Br J Cancer. 58(5), 594-9.
URL:http://www.ncbi.nlm.nih.gov/pubmed/3219269.

Rutteman, G. R.; Withrow, S. J.; MacEwen, E. G. (2001):
Tumors of the mammary gland.
In: Small Animal Clinicla Oncology. / Hrsg. S. J. Withrow and E. G. MacEwen. - Philadelphia, PA: WB Saunders. -. S. 455-477.). -.

Salomon (2008):
Anatomie für die Tiermedizin. 2. -: Enke Verlag. - ISBN:978-3-8304-1075-1.).

Sarli, G.; Preziosi, R.; Benazzi, C.; Castellani, G.; Marcato, P. S. (2002):
Prognostic value of histologic stage and proliferative activity in canine malignant mammary tumors.
J Vet Diagn Invest. 14(1), 25-34.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12680640.

Schafer, K. A.; Kelly, G.; Schrader, R.; Griffith, W. C.; Muggenburg, B. A.; Tierney, L. A.; Lechner, J. F.; Janovitz, E. B.; Hahn, F. F. (1998):
A canine model of familial mammary gland neoplasia.
Vet Pathol. 35(3), 168-77.
URL:http://www.ncbi.nlm.nih.gov/pubmed/9598580.

Schmittgen, T. D.; Lee, E. J.; Jiang, J.; Sarkar, A.; Yang, L.; Elton, T. S.; Chen, C. (2008):
Real-time PCR quantification of precursor and mature microRNA.
Methods. 44(1), 31-8.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18158130.

Schneider, R. (1970):
Comparison of age, sex, and incidence rates in human and canine breast cancer.
Cancer. 26(2), 419-26.
URL:http://www.ncbi.nlm.nih.gov/pubmed/5465470.

Schneider, R.; Dorn, C. R.; Taylor, D. O. (1969):
Factors influencing canine mammary cancer development and postsurgical survival.
J Natl Cancer Inst. 43(6), 1249-61.
URL:http://www.ncbi.nlm.nih.gov/pubmed/4319248.

Silver, I. A. (1966):
The anatomy of the mammary gland of the dog and cat.
J Small Anim Pract. 7(11), 689-96.
URL:http://www.ncbi.nlm.nih.gov/pubmed/6009574.

Sonnenschein, E. G.; Glickman, L. T.; Goldschmidt, M. H.; McKee, L. J. (1991):
Body conformation, diet, and risk of breast cancer in pet dogs: a case-control study.
Am J Epidemiol. 133(7), 694-703.
URL:http://www.ncbi.nlm.nih.gov/pubmed/2018024.

Sorenmo, K. (2003):
Canine mammary gland tumors.
Vet Clin North Am Small Anim Pract. 33(3), 573-96.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12852237.

Sorenmo, K. U.; Kristiansen, V. M.; Cofone, M. A.; Shofer, F. S.; Breen, A. M.; Langeland, M.; Mongil, C. M.; Grondahl, A. M.; Teige, J.; Goldschmidt, M. H. (2009):
Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence.
Vet Comp Oncol. 7(3), 162-72.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19691645.

Sorenmo, K. U.; Rasotto, R.; Zappulli, V.; Goldschmidt, M. H. (2011):
Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms.
Vet Pathol. 48(1), 85-97.
URL:http://www.ncbi.nlm.nih.gov/pubmed/21147765.

Stark, A.; Lin, M. F.; Kheradpour, P.; Pedersen, J. S.; Parts, L.; Carlson, J. W.; Crosby, M. A.; Rasmussen, M. D.; Roy, S.; Deoras, A. N.; Ruby, J. G.; Brennecke, J.; Harvard FlyBase, c.; Berkeley Drosophila Genome, P.; Hodges, E.; Hinrichs, A. S.; Caspi, A.; Paten, B.; Park, S. W.; Han, M. V.; Maeder, M. L.; Polansky, B. J.; Robson, B. E.; Aerts, S.; van Helden, J.; Hassan, B.; Gilbert, D. G.; Eastman, D. A.; Rice, M.; Weir, M.; Hahn, M. W.; Park, Y.; Dewey, C. N.; Pachter, L.; Kent, W. J.; Haussler, D.; Lai, E. C.; Bartel, D. P.; Hannon, G. J.; Kaufman, T. C.; Eisen, M. B.; Clark, A. G.; Smith, D.; Celniker, S. E.; Gelbart, W. M.; Kellis, M. (2007):
Discovery of functional elements in 12 Drosophila genomes using evolutionary signatures.
Nature. 450(7167), 219-32.
URL:http://www.ncbi.nlm.nih.gov/pubmed/17994088.

Stephenson, G. D.; Rose, D. P. (2003):
Breast cancer and obesity: an update.
Nutr Cancer. 45(1), 1-16.
URL:http://www.ncbi.nlm.nih.gov/pubmed/12791499.

Stratmann, N.; Failing, K.; Richter, A.; Wehrend, A. (2008):
Mammary tumor recurrence in bitches after regional mastectomy.
Vet Surg. 37(1), 82-6.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18199060.

Taylor, G. N.; Shabestari, L.; Williams, J.; Mays, C. W.; Angus, W.; McFarland, S. (1976):
Mammary neoplasia in a closed beagle colony.
Cancer Res. 36(8), 2740-3.
URL:http://www.ncbi.nlm.nih.gov/pubmed/1277183.

Thomas, D. B. (1984):
Do Hormones Cause Breast-Cancer.
Cancer. 53(3), 595-604.
URL:<Go to ISI>://WOS:A1984SK48300003.

Tokes, A. M.; Szasz, A. M.; Farkas, A.; Toth, A. I.; Dank, M.; Harsanyi, L.; Molnar, B. A.; Molnar, I. A.; Laszlo, Z.; Rusz, Z.; Kulka, J. (2009):
Stromal matrix protein expression following preoperative systemic therapy in breast cancer.
Clin Cancer Res. 15(2), 731-9.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19147781.

Trimboli, A. J.; Cantemir-Stone, C. Z.; Li, F.; Wallace, J. A.; Merchant, A.; Creasap, N.; Thompson, J. C.; Caserta, E.; Wang, H.; Chong, J. L.; Naidu, S.; Wei, G.; Sharma, S. M.; Stephens, J. A.; Fernandez, S. A.; Gurcan, M. N.; Weinstein, M. B.; Barsky, S. H.; Yee, L.; Rosol, T. J.; Stromberg, P. C.; Robinson, M. L.; Pepin, F.; Hallett, M.; Park, M.; Ostrowski, M. C.; Leone, G. (2009):
Pten in stromal fibroblasts suppresses mammary epithelial tumours.
Nature. 461(7267), 1084-91.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19847259.

Tuohy, J. L.; Milgram, J.; Worley, D. R.; Dernell, W. S. (2009):
A review of sentinel lymph node evaluation and the need for its incorporation into veterinary oncology.
Vet Comp Oncol. 7(2), 81-91.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19453362.

Vail, D. M.; MacEwen, E. G. (2000):
Spontaneously occurring tumors of companion animals as models for human cancer.
Cancer Invest. 18(8), 781-92.
URL:http://www.ncbi.nlm.nih.gov/pubmed/11107448.

van 't Veer, L. J.; Dai, H.; van de Vijver, M. J.; He, Y. D.; Hart, A. A.; Mao, M.; Peterse, H. L.; van der Kooy, K.; Marton, M. J.; Witteveen, A. T.; Schreiber, G. J.; Kerkhoven, R. M.; Roberts, C.; Linsley, P. S.; Bernards, R.; Friend, S. H. (2002):
Gene expression profiling predicts clinical outcome of breast cancer.
Nature. 415(6871), 530-6.
URL:http://www.ncbi.nlm.nih.gov/pubmed/11823860.

van Garderen, E.; van der Poel, H. J.; Swennenhuis, J. F.; Wissink, E. H.; Rutteman, G. R.; Hellmen, E.; Mol, J. A.; Schalken, J. A. (1999):
Expression and molecular characterization of the growth hormone receptor in canine mammary tissue and mammary tumors.
Endocrinology. 140(12), 5907-14.
URL:http://www.ncbi.nlm.nih.gov/pubmed/10579357.

van Rooij, E.; Sutherland, L. B.; Liu, N.; Williams, A. H.; McAnally, J.; Gerard, R. D.; Richardson, J. A.; Olson, E. N. (2006):
A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure.
Proc Natl Acad Sci U S A. 103(48), 18255-60.
URL:http://www.ncbi.nlm.nih.gov/pubmed/17108080.

Varallyay, E.; Burgyan, J.; Havelda, Z. (2008):
MicroRNA detection by northern blotting using locked nucleic acid probes.
Nat Protoc. 3(2), 190-6.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18274520.

Wang, F.; Zheng, Z.; Guo, J.; Ding, X. (2010):
Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor.
Gynecol Oncol. 119(3), 586-93.
URL:http://www.ncbi.nlm.nih.gov/pubmed/20801493.

Wang, L.; Li, L.; Guo, R.; Li, X.; Lu, Y.; Guan, X.; Gitau, S. C.; Wang, L.; Xu, C.; Yang, B.; Shan, H. (2014):
miR-101 promotes breast cancer cell apoptosis by targeting Janus kinase 2.
Cell Physiol Biochem. 34(2), 413-22.
URL:http://www.ncbi.nlm.nih.gov/pubmed/25059472.

Weidner, N.; Semple, J. P.; Welch, W. R.; Folkman, J. (1991):
Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma.
N Engl J Med. 324(1), 1-8.
URL:http://www.ncbi.nlm.nih.gov/pubmed/1701519.

Xie, H.; Lim, B.; Lodish, H. F. (2009):
MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity.
Diabetes. 58(5), 1050-7.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19188425.

Yamagami, T.; Kobayashi, T.; Takahashi, K.; Sugiyama, M. (1996):
Prognosis for canine malignant mammary tumors based on TNM and histologic classification.
J Vet Med Sci. 58(11), 1079-83.
URL:http://www.ncbi.nlm.nih.gov/pubmed/8959655.

Yan, L. X.; Huang, X. F.; Shao, Q.; Huang, M. Y.; Deng, L.; Wu, Q. L.; Zeng, Y. X.; Shao, J. Y. (2008):
MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis.
RNA. 14(11), 2348-60.
URL:http://www.ncbi.nlm.nih.gov/pubmed/18812439.

Yan, X.; Chen, X.; Liang, H.; Deng, T.; Chen, W.; Zhang, S.; Liu, M.; Gao, X.; Liu, Y.; Zhao, C.; Wang, X.; Wang, N.; Li, J.; Liu, R.; Zen, K.; Zhang, C. Y.; Liu, B.; Ba, Y. (2014):
miR-143 and miR-145 synergistically regulate ERBB3 to suppress cell proliferation and invasion in breast cancer.
Mol Cancer. 13, 220.
URL:http://www.ncbi.nlm.nih.gov/pubmed/25248370.

Zhang, B. H.; Pan, X. P.; Cobb, G. P.; Anderson, T. A. (2007):
microRNAs as oncogenes and tumor suppressors.
Developmental Biology. 302(1), 1-12.
URL:<Go to ISI>://WOS:000243862900001.

Zhang, X. C.; Piccini, A.; Myers, M. P.; Van Aelst, L.; Tonks, N. K. (2012):
Functional analysis of the protein phosphatase activity of PTEN.
Biochemical Journal. 444, 457-464.
URL:<Go to ISI>://WOS:000305545300010.

Zhang, Y.; Gao, J. S.; Tang, X.; Tucker, L. D.; Quesenberry, P.; Rigoutsos, I.; Ramratnam, B. (2009):
MicroRNA 125a and its regulation of the p53 tumor suppressor gene.
FEBS Lett. 583(22), 3725-30.
URL:http://www.ncbi.nlm.nih.gov/pubmed/19818772.

8 PUBLICATION LIST

Talks:

Marie-Charlotte von Deetzen, Bernd Schmeck, Achim D. Gruber, Robert Klopfleisch:
Malignitäts-assoziierte Expressionsunterschiede von mikroRNA-Molekülen bei kaninen Mammatumoren.
55 th Meeting of the Pathology Group of the German Veterinary Society, Fulda, Germany, 10.-11.03.2012

Marie-Charlotte von Deetzen, Bernd Schmeck, Achim D. Gruber, Robert Klopfleisch:
Malignitäts-assoziierte Expressionsunterschiede von mikroRNA-Molekülen bei kaninen Mammatumoren.
Ph.D. Symposium of the Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany, 13.07.2012

Poster presentations:

Marie-Charlotte von Deetzen, Bernd Schmeck, Achim D. Gruber, Robert Klopfleisch:
Vergleich verschiedener Extraktionsverfahren für den Nachweis von mikroRNA.
Ph.D. Symposium of the Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany, 01.07.2011

Paper:

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Journal: Research in Veterinary Science 95 (2013) 562–568

von Deetzen M-C, Schmeck B, Gruber AD, Klopfleisch R:
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Selbständigkeitserklärung

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

Oldenburg, 16.12.2014

Marie-Charlotte von Deetzen