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Canine mammary tumours:
new insights into prognosis and molecular classification

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Abstract

In canine species, spontaneous mammary tumours constitute the second most frequent neoplasia, surpassed only by skin tumours. When considering female dogs, mammary tumours represent the most common neoplasia, with malignant tumours accounting for up to 50% of cases. These facts have raised an increasing interest on the research of reliable prognostic factors in canine mammary tumours, and similarly to humans, the veterinary pathologist might assume a fundamental role by providing both histological diagnosis as well as additional information regarding the prognosis of a particular animal.

At present, and despite several prognostic studies in this area, results are not consensual, which is mandatory for the validation of classical clinicopathological parameters and the search of novel prognostic factors. Therefore, the central goal of our thesis was the research of clinicopathological and molecular factors with potential impact on the prognosis of canine mammary tumours.

The present thesis is composed by seven chapters: an initial chapter (Chapter I) corresponding to the state of the art; Chapters II-VI, which correspond to scientific articles resulting from our investigation; and Chapter VII, which promotes a global and final discussion of the results.

Chapter I (General Introduction) is a review of the most recent literature concerning canine mammary tumours, especially with regard to prognostic studies. A particular emphasis is given to several molecular cell markers, in view of both canine and human scientific literature. At the end of this chapter, we have delineated the main goals of the present thesis.

In Chapter II (Canine mammary gland tumours: clinical and pathological parameters as predictors of overall and disease-free survival - a univariate and multivariate analysis), we have performed a clinical and histopathological characterization of a hundred and fifty six canine mammary tumour specimens (46 benign and 110 malignant). In order to investigate the prognostic value of clinical and pathological variables, a follow-up study was performed in 69 female dogs for a minimum period of 12 months after surgical procedure. Univariate analysis showed that tumour size, histological type, tumour growth, differentiation grade, stromal and lymphovascular invasion, lymph node status, mitotic and Ki-67 labelling indices were significantly associated with overall and

disease-free survival. Skin ulceration was only associated with poorer overall survival rates. Cox regression multivariate analysis revealed lymph node status as the only independent prognostic factor.

Chapter III (Expression of E-cadherin, P-cadherin and β-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival) describes the immunohistochemical evaluation of several adhesion molecules on a series of 65 canine malignant mammary tumours. Given the critical role assigned to cadherin-mediated cell adhesion during embryogenesis and in the maintenance of normal adult tissue architecture, as well as its putative involvement in tumour cell invasion and progression, we sought to investigate their expression in canine malignant mammary tumours and their association with clinicopathological variables, proliferation and survival.

Reduction in E-cadherin expression was significantly associated with increased tumour size, high histological and invasion grades, lymph node metastasis and high mitotic index, whereas reduced β-catenin expression was associated with high histological and invasion grades. P-cadherin expression was only associated with invasion. In 39 cases for which follow-up data was available, reduced E-cadherin and β-catenin expression was significantly associated with shorter overall survival and disease-free survival. Although this study has been performed with a relatively small number of cases, we have observed that an abnormal expression of adhesion molecules is a common phenomenon in canine mammary malignant tumours and, therefore, may play a central role in tumour progression. Further studies with a larger series will certainly highlight the prognostic value of these molecules in the context of canine mammary tumours.

In Chapter IV (Immunohistochemical expression of Epidermal Growth Factor Receptor (EGFR) in canine mammary tissues), an evaluation of EGFR immunohistochemical expression was performed in a series of 136 canine mammary tumours (46 benign and 90 malignant) and representative areas of adjacent normal and hyperplastic mammary tissue. Despite the availability of several biochemical studies of EGFR in canine mammary tumours, there are still no immunohistochemical studies concerning its expression, which directed us to its evaluation both in benign and malignant tumours. Immunohistochemistry has the advantage of disclosing the precise cellular location of a particular protein, which is not possible by using immunoenzymatic methodologies.

In normal and hyperplastic canine mammary glands, EGFR expression was consistently observed in myoepithelial cells, with luminal cells usually negative. Perilobular stroma was commonly positive. In benign tumours, EGFR was present in both epithelial cell components, but luminal cells were weakly positive, when compared to malignant tumours. In fact, EGFR overexpression was found in 9 benign (19.6%) and 38 malignant (42.2%) lesions, with EGFR positivity significantly related with malignancy. Besides animal age and tumour size, there were no significant associations between other clinicopathological parameters and EGFR overexpression. On survival analysis, tumours with EGFR overexpression showed a reduced disease-free and overall survival; however, these associations failed to reach statistically significant levels. Further studies are warranted, namely concerning the analysis of EGFR gene amplification, given that EGFR might represent a potential therapeutic target.

Chapter V (Expression and prognostic significance of cytokeratin (CK) 19 in canine malignant mammary tumours) describes the immunohistochemical evaluation of CK19 in a series of 102 malignant canine mammary tumours and investigates the possible association between CK19 pattern of expression and clinicopathological parameters, proliferation and survival. This study was based on recent evidence demonstrating a significant association between the reduction of luminal CK (such as CK19) and a more aggressive behaviour of human breast cancer, usually associated with a basal phenotype. Therefore, besides the evaluation of the prognostic potential of this luminal cell marker, we have also investigated its association with a basal/myoepithelial phenotype, by using additional specific cell differentiation markers.

Reduced/absent CK19 was significantly associated with histological type, invasiveness, high histological grade and an elevated Ki-67 index. CK19 positive expression was significantly associated with the presence of ER, whereas its reduced immunostaining was associated with basal/myoepithelial cell markers positive expression. Survival analysis demonstrated that down-regulation of this luminal CK is significantly associated with shorter overall and disease-free survival rates; however, CK19 was not an independent prognostic factor in multivariate analysis. In our series, CK19 down-regulation was significantly related to an aggressive phenotype; yet, the real implication of this phenomenon is not known, namely during tumour progression.

Chapter VI (Identification of molecular phenotypes in canine mammary carcinomas with clinical implications: application of the human classification) illustrates the application of a recently described classification for human breast carcinomas to a series

of 102 canine mammary carcinomas. This classification was initially based on gene expression profiling analysis and it was later on reinforced at the protein level, by using immunohistochemistry; both methodologies identified distinct phenotypes of human breast cancer associated with distinct clinical behaviours. Similarly to human studies, by using an immunohistochemistry surrogate panel based on five molecular markers (estrogen receptor, HER-2, cytokeratin 5, p63 and P-cadherin), we were able to classify canine mammary carcinomas into four different subtypes: luminal A (ER+/HER-2-), luminal B (ER+/HER-2+), basal (ER-/HER-2- and a basal marker positive) and HER-2 overexpressing tumours (ER-/HER-2+).

Luminal A-type tumours were characterized by lower grade and proliferation rate, whereas basal-type tumours were mostly high grade, high proliferative and positive for CK5, p63 and P-cadherin. In addition, as in humans, basal subtype was significantly associated with shorter disease-free and overall survival rates.

Although we consider these findings as preliminary results, which require further validation, this study pointed out to similar phenotypes to the ones described in the human literature. So, canine mammary carcinomas might represent a suitable natural model for the study of human breast carcinomas, in particular to the basal subset, given the putative high percentage of basal carcinomas identified in the dog.

The final Chapter (Chapter VII – General discussion and concluding remarks) encloses a global discussion of our investigation, stressing the most relevant and significant findings.

Resumo

Na espécie canina, os tumores mamários espontâneos representam a segunda neoplasia mais comum, sendo apenas ultrapassados pelos tumores de pele. Considerando os indivíduos do sexo feminino, os tumores de mama constituem a neoplasia espontânea mais frequente, representando os tumores malignos cerca de 50% dos casos observados. Estes factos suscitam um interesse crescente na pesquisa de factores de prognóstico credíveis na área dos tumores mamários caninos e à semelhança do que ocorre em Medicina Humana, o patologista veterinário pode assumir um papel fundamental ao fornecer não apenas um diagnóstico histológico, como também informação adicional acerca do prognóstico de um determinado indivíduo.

Actualmente, e apesar de vários estudos de prognóstico nesta área, os resultados não são consensuais pelo que se torna necessária a validação dos parâmetros clínico-patológicos considerados clássicos e a pesquisa de novos factores com valor prognóstico. Assim, tendo como objectivo central a pesquisa de factores com possível impacto no prognóstico dos tumores de mama de cadela, procedemos ao estudo de diversas características clínico-patológicas e moleculares, que se encontram discriminadas ao longo deste trabalho.

A presente dissertação é constituída por sete capítulos: um capítulo inicial de revisão bibliográfica (Capítulo I); os Capítulos II a VI, que correspondem aos artigos científicos resultantes da investigação desenvolvida; e o Capítulo VII, onde se promove uma discussão geral do trabalho efectuado.

O Capítulo I (Introdução Geral) consiste numa revisão bibliográfica actualizada acerca dos tumores de mama de cadela, em especial no que diz respeito a estudos de prognóstico. É ainda dado ênfase particular a alguns marcadores moleculares utilizados ao longo do nosso trabalho, tendo em consideração estudos efectuados em tumores mamários caninos e humanos. No fim deste capítulo, são enumerados os objectivos da presente dissertação.

Ao longo do Capítulo II (Tumores mamários caninos: parâmetros clínico-patológicos como factores preditivos da sobrevivência total e sobrevivência livre de doença – análise uni- e multivariada) procedeu-se à caracterização clínica e histopatológica de uma série de 156 tumores de mama de cadela (46 benignos e 110 malignos). Com o objectivo de investigar o valor prognóstico de variáveis clínico-patológicas, foi

efectuado um estudo de sobrevivência após exérese cirúrgica em 69 animais, durante um período mínimo de 12 meses. A análise univariada revelou que o tamanho do tumor, o tipo histológico, o modo de crescimento, o grau histológico, a invasão estromal e linfo-vascular, a presença de metástases ganglionares, e os índices de proliferação se encontravam significativamente associados com as sobrevivências total e livre de doença. A presença de ulceração cutânea encontrou-se associada apenas com a sobrevida total. A análise multivariada revelou a presença de metástases ganglionares como o único factor de prognóstico independente.

No Capítulo III (Expressão da caderina E, caderina P e β-catenina em tumores mamários caninos malignos em relação a parâmetros clínico-patológicos, proliferação e sobrevivência) efectuou-se a avaliação imunohistoquímica de moléculas de adesão numa série de 65 tumores mamários malignos de cadela. Tendo em conta vários estudos que demonstram a função importante da adesão mediada por caderinas durante os processos de desenvolvimento e na manutenção da arquitectura dos tecidos adultos, bem como o seu envolvimento durante a invasão e progressão tumoral, investigámos a expressão das moléculas acima descritas em tumores mamários malignos de cadela e a sua possível associação com parâmetros clínico-patológicos clássicos, índices de proliferação e sobrevivência.

Observámos que a redução da expressão da caderina E esteve significativamente associada com o tamanho do tumor, alto grau histológico, invasão, presença de metástases ganglionares e elevado índice mitótico; por outro lado, a redução da expressão da β-catenina encontrou-se significativamente associada com alto grau histológico e invasão. Relativamente à caderina P, a sua expressão encontrou-se significativamente associada apenas com a invasão. No que diz respeito ao estudo de sobrevivência, a redução da caderina E e β-catenina encontrou-se significativamente associada com menor tempo de sobrevivência total e livre de doença. Apesar deste estudo ter sido efectuado com um número reduzido de amostras, observou-se que a expressão alterada do complexo caderina-catenina é um evento comum nestas neoplasias. A realização de novos estudos com maior número de casos irá certamente esclarecer o valor prognóstico destas moléculas no contexto dos tumores mamários caninos.

No Capítulo IV (Expressão imunohistoquímica do Receptor para o Factor de Crescimento Epidérmico (*EGFR*) em tecidos mamários caninos), descreveu-se a

avaliação do EGFR através da técnica de imunohistoquímica numa série de 136 tumores mamários caninos (46 benignos e 90 malignos). Avaliou-se ainda a sua expressão na glândula mamária normal e hiperplásica adjacente. Apesar da existência de vários trabalhos em tumores mamários caninos com recurso a métodos imunoenzimáticos para a avaliação do EGFR, não existem ainda estudos de imunohistoquímica, pelo que considerámos importante avaliar a sua expressão em tumores benignos e malignos, nomeadamente a sua localização celular, informação que não é disponibilizada recorrendo às metodologias previamente descritas na literatura.

Na glândula mamária canina normal e hiperplásica, a expressão do EGFR foi observada principalmente ao nível das células mioepiteliais. No entanto, detectou-se positividade para este receptor em algumas células epiteliais luminais ductais, assim como no estroma perilobular. Relativamente aos tumores benignos, o EGFR foi observado no componente epitelial e mioepitelial, apresentando as células epiteliais um nível de expressão reduzido, quando comparado com os tumores malignos. De facto, a expressão de EGFR encontrou-se significativamente associada com a malignidade tumoral, tendo sido detectada uma imunoexpressão membranar completa de EGFR em mais de 10% das células neoplásicas em 42.2% de tumores malignos, versus 19.6% tumores benignos. Não se observou qualquer associação entre a expressão neoplásica do EGFR e os parâmetros clínico-patológicos, à excepção da idade e do tamanho do tumor. Apesar da sobre-expressão do EGFR mostrar uma tendência para um pior prognóstico, não foram encontradas associações estatisticamente significativas neste estudo. Acreditamos serem necessários estudos futuros acerca deste receptor, nomeadamente analisando a presença de amplificação do gene *EGFR,* já que este receptor pode constituir um potencial alvo terapêutico.

No Capítulo V (Expressão e valor prognóstico da citoqueratina (CK) 19 em tumores mamários malignos da cadela) procedeu-se à avaliação imunohistoquímica da CK19 numa série de 102 tumores mamários malignos de cadela, analisando-se a possível associação entre o seu padrão de expressão e parâmetros clínico-patológicos, proliferação e tempos de sobrevivência. À luz de estudos recentes em carcinomas humanos que demonstram uma associação entre a redução da expressão de CK luminais e uma maior agressividade biológica, julgámos pertinente investigar o padrão de expressão da CK19 (CK luminal) nos tumores mamários malignos da cadela, nomeadamente o seu potencial valor prognóstico e também a sua possível associação a

um fenótipo basal/mioepitelial, para tal utilizando marcadores específicos de diferenciação celular.

Neste trabalho, observámos que a redução ou ausência da CK19 se encontrou significativamente associada com o tipo histológico, invasão, alto grau histológico e índice Ki-67 elevado. A expressão da CK19 encontrou-se significativamente associada com a presença de receptores de estrogénio (ER), enquanto a sua redução se revelou associada com a presença de marcadores basais/mioepiteliais. Relativamente ao estudo de sobrevivência, a redução ou ausência da expressão deste marcador luminal provou estar associada a menores tempos de sobrevivência; no entanto, a CK19 não foi considerada como factor de prognóstico independente em análise multivariada. Apesar de, neste estudo, a ausência ou redução da expressão da CK19 se encontrar associada a um fenótipo tumoral mais agressivo, o significado biológico deste achado relativamente à progressão neoplásica permanece por esclarecer.

O Capítulo VI (Identificação de fenótipos moleculares em carcinomas mamários caninos com implicação clínica: aplicação de uma classificação humana) reflecte a tentativa de aplicação de uma classificação recentemente descrita para os carcinomas mamários humanos a uma série de 102 carcinomas mamários caninos. Esta classificação teve como base estudos de expressão genética e foi posteriormente comprovada através da técnica de imunohistoquímica, distinguindo diferentes fenótipos moleculares de cancro de mama humano. Recorrendo a marcadores válidos em Medicina Humana para a sua identificação (ER, HER-2, CK5, p63 and caderina P), classificámos os carcinomas mamários caninos em 4 subtipos principais: luminal A (ER+, HER-2-), luminal B (ER+, HER-2+), basal (ER-, HER-2- e um marcador basal positivo) e HER-2 (ER-, HER-2+).

À semelhança da mulher, os tumores classificados como luminal A apresentaram baixo grau histológico e menores índices de proliferação, enquanto os carcinomas "basais" se caracterizaram geralmente por alto grau histológico e elevados índices de proliferação. Quanto à sobrevivência, também nos carcinomas de mama de cadela observámos uma associação entre o fenótipo basal e tempos de sobrevivência menores. Estes resultados parecem apontar para a existência de fenótipos moleculares semelhantes aos descritos em Medicina Humana, sugerindo o carcinoma de mama da cadela como um potencial modelo natural de estudo para o carcinoma de mama da mulher.

No Capítulo VII (Discussão geral e conclusões) procede-se à discussão global do trabalho desenvolvido, evidenciando-se os seus aspectos mais relevantes.

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List of Abbreviations and Symbols

AgNORs	Silver-stained nucleolar organizer regions
BRCA	*Breast Cancer*
Ca	Calcium
cat	catenin
CD	Cluster of differentiation
CECAV	Centro de Ciência Animal e Veterinária
CI	Confidence interval
CK	Cytokeratin
Cm	centimetre
DAB	3,3-diaminobenzidine tetrahydrochloride
DFS	Disease-free survival
DNA	Deoxyribonucleic Acid
E-cadherin	Epithelial cadherin
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Oestrogen Receptor
ERE	Oestrogen responsive element
ERK	Extracellular signal regulated kinase
FDA	Food and Drug Administration
Fig.	Figure
GPR	G protein coupled receptor
HE	Haematoxylin and Eosin
HER	Human Epidermal Growth Factor Receptor
HR	Hazard ratio
H_2O_2	Hidrogen Peroxide
IGF-1 R	Insulin Growth Factor-1 Receptor
IPATIMUP	Instituto de Patologia e Imunologia Molecular da Universidade do Porto
kDa	KiloDalton
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metalloproteinase
µm	micrometre
Max	Maximum
Min	Minimum
min	minute
mg	milligram
mL	millilitre
mM	milliMolar

mm^2	square millimetre
mRNA	Messenger Ribonucleic Acid
OS	Overall survival
PBS	Phosphate buffered saline
P-cadherin	Placental cadherin
PCNA	Proliferating cell nuclear antigen
PI3K	Phosphatidylinositol 3 kinase
PR	Progesterone Receptor
SMA	Smooth muscle actin
SPSS	Statistical Package for the Social Sciences
TA	Transactivation
TF	Transcription factor
TGF	Transforming Growth Factor
USA	United States of America
WHO	World Health Organization

Chapter I

General Introduction

1. CANINE MAMMARY GLAND TUMOURS

General considerations

Similarly to rodents, felines and humans, canine mammary gland is frequently affected by spontaneous tumours, which represent the second most frequent neoplasia (Ferguson, 1985; Madewell and Theilen, 1987; Moulton, 1990). Spontaneous mammary gland tumours occur almost exclusively in female dogs, representing the most commonly occurring neoplasm and accounting for 25 to 50% of all neoplasias (Moulton, 1990; Misdorp *et al*., 1999, 2002; Sorenmo, 2003). The exact incidence of these tumours is difficult to determine but it has been estimated in 105:100.000, which is three times higher compared to women (Brodey *et al*., 1983).

Canine mammary tumours mainly affect middle-aged bitches (Loar, 1989; Moulton, 1990; Hellmén, 1996; Rutteman *et al*., 2001), occur more often in caudal mammary glands and are clinically manifested as single or multiple nodules. Purebred dogs, namely spaniel breeds, pointers and dachshunds seem to be predisposed (Rutteman *et al*., 2001; Misdorp, 2002).

Based on histological and biological criteria, it can be estimated that approximately one third to half of the surgically removed canine mammary tumours are malignant (Misdorp, 2002). Therefore, this disease represents a serious problem in worldwide veterinary practice and is a matter of concern for both oncologists and pathologists, which is ultimately reflected on the escalating number of studies in this research area. Furthermore, canine mammary tumours have attracted considerable attention over the years as possible animal models for human mammary neoplasia, based on their morphological and biological similarities (Gilbertson *et al*., 1983).

Etiopathogenesis

Tumourigenesis is a multistep process comprising initiation, promotion and progression. The initiation of breast cancer is due to transforming (genetic and epigenetic) events in a single cell. Promotion and subsequent tumour progression are driven by the accumulation of additional genetic changes combined with clonal expansion and selection (Beckmann *et al*., 1997; Porter *et al*., 2001). So, invasive

mammary cancer is the endpoint of a multiple-step evolution that can be tracked as a series of progressive histological and molecular lesions. Under the microscope we can occasionally recognize the progression of hyperplasia, in situ carcinoma and invasive cancer, however the molecular evolution is less clearly understood (Ross, 1998).

The etiopathogenesis of canine mammary tumours is still unclear, despite several reported genetic alterations concerning oncogenes (Ahern *et al*., 1996; Rungsipipat *et al*., 1999; Martin de las Mulas *et al*., 2003), tumour suppressor genes (Van Leeuwen *et al*., 1996; Chu *et al*., 1998; Veldhoen *et al*., 1999) and the breast cancer susceptibility gene *BRCA*1 (Yuzbasiyan-Gurkan *et al*., 1999). In addition, gross abnormalities in the nuclear DNA content (DNA aneuploidy) have been found in 50 to 60% of canine malignant mammary tumours. This aneuploidy reflects genetic instability, which is commonly at the basis of malignant transformation. In fact, some benign tumours were also found aneuploid, possibly reflecting their potential to progress to malignancy (Rutteman *et al*., 1988a; Hellmén *et al*., 1993; Rutteman *et al*., 2001).

Human breast cancer represents a complex disease modulated by host factors, such as the hormonal status, which is involved in breast tumour development and progression (Ross, 1998). The participation of steroid receptors in the development of canine mammary tumours is not fully understood. It is known that early ovariohysterectomy offers a considerable protective effect, with the risk of developing mammary tumours increasing from 0.5% to 8%, and to 26%, depending on whether the ovariohysterectomy is performed before the first, second, or any oestrus thereafter, respectively (Schneider *et al*., 1969). Moreover, the administration of steroid hormones and their synthetic derivatives (progestins or progestin-estrogen combinations at high dosage) was found to promote the formation of mammary tumours in the dog (Misdorp *et al*., 1988; Stovring *et al*., 1997; Rutteman *et al*., 2001; Misdorp, 2002). It was also shown that mechanisms involved in progesterone-induced mammary gland tumours include an upregulation of growth hormone production within the mammary gland, where it has a direct growth stimulatory effect (Selman *et al*., 1994; Mol *et al*., 1999). Taken together, these findings suggest that steroid hormones might play an important role in the pathogenesis of canine mammary tumours (Sorenmo *et al*., 2000).

As in humans, advancing age, obesity and diet also seem to increase the risk of mammary tumours in the dog (Perez Alenza *et al*., 2000), but a protective effect of early pregnancy has not been demonstrated (Rutteman *et al*., 2001).

Pathology and natural behaviour

The classification of canine mammary neoplasms has been based mainly on standard histopathology (descriptive morphology), and to a lesser extent on the histogenetic origin and prognosis (Fowler *et al*., 1974; Hampe and Misdorp, 1974; Destexhe *et al*., 1993b; Benjamin *et al*., 1999; Misdorp *et al*., 1999; Misdorp, 2002). Canine mammary tumours are characterized by a complex morphology forming epithelial, mixed and mesenchymal tumours. All types can exist as benign and malignant forms: complex adenoma and mixed benign tumours constitute the dominant benign histotypes, whereas carcinomas represent the majority of malignant tumours. True malignant mixed tumours (carcinosarcomas) and sarcomas do exist but are uncommon (Misdorp *et al*., 1999; Hellmén *et al*., 2000). The current WHO classification of canine mammary tumours (Table 1) is both descriptive and prognostic, and subdivides carcinomas into noninfiltrating carcinomas, complex carcinomas (two cell types) and simple carcinomas (one cell type), in an attempt to rank the tumours by increasing malignant potential (Misdorp *et al*., 1999).

Table 1. Histological classification of canine mammary tumours (Misdorp *et al*., 1999).

HISTOLOGICAL CLASSIFICATION OF CANINE MAMMARY TUMOURS	
BENIGN TUMOURS	Adenoma
	Simple adenoma
	Complex adenoma
	Basaloid adenoma
	Fibroadenoma
	Low-cellularity fibroadenoma
	High-cellularity fibroadenoma
	Benign mixed tumour
	Duct papilloma
MALIGNANT TUMOURS	Noninfiltrating (in situ) carcinoma
	Complex carcinoma
	Simple carcinoma
	Tubulopapillary carcinoma
	Solid carcinoma
	Anaplastic carcinoma
	Special types of carcinomas
	Spindle cell carcinoma
	Squamous cell carcinoma
	Mucinous carcinoma
	Lipid-rich carcinoma
	Sarcoma
	Fibrosarcoma/ Osteosarcoma/ Other sarcomas
	Carcinosarcoma
	Carcinoma or sarcoma in benign tumour

Between 41 and 53% of all mammary tumours that occur in the female dog are considered malignant (Brodey *et al*., 1983; Gilbertson *et al*., 1983; Misdorp *et al*., 1999; Moe, 2001; Rutteman *et al*., 2001; Hellmén, 2005). Small, non-invasive, well-differentiated tumours are often treated effectively with surgery alone, but dogs with large, invasive, or poorly differentiated tumours are at risk of developing metastasis and dying of the disease. Approximately 30% of carcinomas cause metastases, usually via the lymphatics to the regional lymph nodes and the lungs, whereas more than 75% of sarcomas give rise to metastases, usually by the haematogenous route (Sorenmo, 2003; Hellmén, 2005). Nevertheless, considerable variations are observed in the biological behaviour among canine mammary tumours and histomorphological evidence of malignancy does not invariably imply a malignant clinical course (Rutteman *et al*., 2001).

Histogenesis and differentiation

Canine mammary tumours are known for their biological and morphological heterogeneity (Nerurkar *et al*., 1989; Moulton, 1990) and their precise histogenesis (especially of mixed tumours) has challenged veterinary pathologists ever since the early days of diagnostic pathology.

Mammary gland has a tubulo-alveolar structure composed of two cell layers, an inner luminal cell layer composed of glandular epithelial cells, and a distinct outer basal cell layer, juxtaposed to the basement membrane (Fig. 1A), composed of spindle-shaped or cuboidal myoepithelial cells, depending on their location and the hormonal status (Gusterson *et al*., 2005). These cells have a common origin, arising from progenitor cells located in a suprabasal compartment between the luminal and the basal layer (Boecker *et al*., 2002; Boecker and Buerger, 2003; Birnbaum *et al*., 2004). It was shown that cells seem to exist at intermediate state of maturation, in both the epithelial and myoepithelial cell lineage (Boecker and Buerger, 2003).

There is overwhelming evidence that virtually all tumours are clonal and represent the progeny of a single cell. What is less clear is which cells within the tumour clone possess tumour-initiating cell function and are capable of maintaining cell growth (Dick, 2003). Substantial data suggest that both stem and progenitor cells may be the

targets of transformation during tumourigenesis, leading to breast cancer heterogeneity (Dontu *et al*., 2004; Kalirai and Clarke, 2006).

Despite rare in the human breast, mixed mammary tumours are very frequent lesions in dogs and show many histological similarities with human pleomorphic adenomas of salivary glands (Genelhu *et al*., 2007). These tumours are characterized by the proliferation of an epithelial and a mesenchymal component, and a number of studies have addressed the histogenesis of metaplastic elements, like cartilage and bone (Misdorp *et al*., 1999). Besides glandular cells, morphologically different types of myoepithelial cells are observed, and several authors favoured a myoepithelial cell role in this tumour type histogenesis, based on immunohistochemical, electron microscopy and cell line studies (Pulley, 1973; Fowler *et al*., 1974; Tateyama and Cotchin, 1977, 1978; Destexhe *et al*., 1993b; Griffey *et al*., 1993; Arai *et al*., 1995; Gärtner *et al*., 1999; Misdorp *et al*., 1999; Tateyama *et al*., 2001; Espinosa de Los Monteros *et al*., 2002; Gama *et al*., 2003; Ramalho *et al*., 2006). Similarly, some human studies have also disclosed a myoepithelial cell histogenesis for benign pleomorphic adenoma of salivary glands (Erlandson *et al*., 1984). However, others have refuted this hypothesis and have supported an epithelial (Monlux *et al*., 1977), stromal (Palmer and Monlux, 1979; Nerurkar *et al*., 1989; Vos *et al*., 1993) or, more recently, a stem cell ontogeny (Hellmén and Lindgren, 1989; Hellmén *et al*., 2000) for canine mixed neoplasms. As for human metaplastic carcinomas (which include mixed malignant tumours), several lines of evidence favour a monoclonal origin for both epithelial and mesenchymal elements (Thompson *et al*., 1996; Zhuang *et al*., 1997; Wada *et al*., 1998), and a number of studies support a basal/myoepithelial histogenesis or differentiation (Sapino *et al*., 1992; Reis-Filho *et al*., 2003; Leibl *et al*., 2005).

Human breast cancers, as determined morphologically, were thought to arise exclusively from the inner, luminal epithelial cell compartment of the terminal-duct lobular unit of the breast. Irrespective of the true histogenesis of breast carcinoma, it has become increasingly clear that a small proportion of cancers (up to 18%) exhibit a partial or complete basal/myoepithelial phenotype, meaning they express molecules normally seen in the basal/myoepithelial compartment of the normal breast (Zhuang *et al*., 1997; Tsuda *et al*., 2000; Lakhani and O´Hare, 2001).

Although mixed tumours are rather frequent canine benign mammary lesions, the malignant counterpart is uncommon. However, myoepithelial differentiation is a

frequent finding in canine mammary malignant tumours, accompanied by the proliferation of glandular epithelial cells in the so-called complex carcinomas. Other frequent carcinomas are of simple type, usually thought to arise from luminal epithelial cells. Nevertheless, as in humans, several immunohistochemical studies pointed out to the presence of a basal/myoepithelial cell phenotype in a subset of simple carcinoma cases, which was not readily recognizable by routine histological evaluation only (Destexhe *et al*., 1993b; Griffey *et al*., 1993; Gama *et al*., 2003). This "basal" differentiation has raised the attention from pathologists, since these were high grade tumours that presented an aggressive behaviour and poor patient prognosis (Griffey *et al*., 1993; Tsuda *et al*., 2000; Jones *et al*., 2001; Laakso *et al*., 2005).

In human breast cancer studies, this intriguing phenotype has reemerged in the past few years, due to the introduction of high-throughput technologies. Recent gene expression cDNA microarray studies have made it possible to distinguish two major tumour classes of breast cancer: one with the characteristics of basal/myoepithelial and the other with the characteristics of luminal cells (Perou *et al*., 2000). Of major importance is the prognostic significance of basal-like cancers, which are frequently associated with poor clinical outcome. Basal-like tumours are hormonal receptor negative and express genes characteristic of basal and myoepithelial cells (Sorlie *et al*., 2001, 2003; Sotiriou *et al*., 2003).

Prognosis

As in humans, the identification of parameters with prognostic relevance constitutes a major area of investigation in canine mammary cancer (Bratulic *et al*., 1996; Lohr *et al*., 1997; Funakoshi *et al*., 2000; Geraldes *et al*., 2000; Nieto *et al*., 2000) and in the last years an increasing number of potential prognostic factors (clinicopathological and molecular factors) have been investigated.

Although not consensual, several clinicopathological features have been recognized as prognostic factors in the vast majority of canine mammary cancer studies, based on univariate and/or multivariate analysis: tumour size (Bostock, 1975; Misdorp and Hart, 1976; Yamagami *et al*., 1996b; Chang *et al*., 2005; Martin de las Mulas *et al*., 2005), ulceration (Hellmén *et al*., 1993; Peña *et al*., 1998; Queiroga and Lopes, 2002), tumour histological type (Misdorp and Hart, 1976; Hellmén *et al*., 1993; Chang *et al*., 2005)

and grade (Karayannopoulou *et al*., 2005; Martin de las Mulas *et al*., 2005), degree of invasion (Bostock, 1975; Misdorp and Hart, 1976; Gilbertson *et al*., 1983; Hellmén *et al*., 1993; Martin de las Mulas *et al*., 2005), presence of lymph node and distant metastasis (Hellmén *et al*., 1993; Yamagami *et al*., 1996b; Queiroga and Lopes, 2002; Philibert *et al*., 2003; Chang *et al*., 2005).

Tumour size has been found to be an independent prognostic factor in a number of studies, with tumours smaller than 3 cm in diameter associated with a significantly better prognosis (Misdorp and Hart, 1976; Yamagami *et al*., 1996b).

Tumour type was found to be an important factor in several studies, which described a range of increasing malignancy from complex carcinoma to simple carcinoma to sarcoma (Misdorp and Hart, 1976; Hellmén *et al*., 1993; Philibert *et al*., 2003). Carcinomas with myoepithelial cell proliferation (complex carcinomas) are usually associated with longer survival times, and although the mechanisms still remain unclear, this fact may be related to the putative role of myoepithelial cells as natural invasion tumour suppressors (Yamagami *et al*., 1996b). Within the group of simple carcinomas, an increasing order of malignancy was observed from tubulopapillary to solid to anaplastic carcinoma (Bostock, 1975; Misdorp *et al*., 1999). Carcinosarcomas were also associated with poor prognosis, with most dogs developing metastasis within the first year after surgery (Benjamin *et al*., 1999).

Most studies found histological grade as being a reliable prognostic factor in canine mammary tumours (Misdorp and Hart, 1976; Gilbertson *et al*., 1983; Peña *et al*., 1998; Benjamin *et al*., 1999). Initial criteria proposed for histological grading of canine mammary carcinomas by Misdorp and Hart (1976) and Gilbertson *et al*. (1983) were based on a combination of rather subjective cellular features. Recently, Karayannopoulou *et al*. (2005) applied the Elston and Ellis human grading method for histological grading and found it predictive for dog mammary tumours. This method (based on tubule formation, nuclear pleomorphism and mitotic count evaluation) is apparently more consistent and shows reproductive results (Elston and Ellis, 1998).

Factors that do not seem to be associated with prognosis are breed, tumour location, number of tumours and type of surgery (as long as histologically adequate resection is achieved) (Schneider *et al*., 1969; Misdorp and Hart, 1976; Hellmén *et al*., 1993; Yamagami *et al*., 1996b; Rutteman *et al*., 2001). Controversial findings exist regarding animal age and ovariohysterectomy at the time of surgery. Some studies state that

older animals are associated with poorer survival (Schneider *et al*., 1969; Hellmén *et al*., 1993; Peña *et al*., 1998) while others report no statistical influence of age on survival (Hellmén *et al*., 1993; Queiroga and Lopes, 2002; Philibert *et al*., 2003; Chang *et al*., 2005). As for ovariectomy, two distinct groups demonstrated that dogs spayed at the time of surgery survived longer than intact dogs (Sorenmo *et al*., 2000; Chang *et al*., 2005), in contrast to other studies reporting no effect of simultaneous ovariectomy on survival (Yamagami *et al*., 1996a; Morris *et al*., 1998; Philibert *et al*., 2003).

Classical clinicopathological factors are not always sufficient to predict the biological behaviour of canine mammary tumours and the availability and application of new methodologies have allowed the identification of new prognostic factors, some potentially relevant as therapeutic targets. Next, we will focus on some molecular factors with potential prognostic value in canine mammary cancer.

2. DIFFERENTIATION AND PROGNOSTIC MARKERS IN MAMMARY CANCER: FROM DOGS AND HUMANS

Differentiation cell markers in mammary cancer

Mammary epithelial cells can be recognized by their distinct immunoprofile: luminal epithelial cells are characterized by the expression of luminal cytokeratins (CK) 7, 8, 18 and 19, whereas basal/myoepithelial cells express basal CK 5, 14 and 17, p63, P-cadherin, CD10 and EGFR, among other markers (Malzahn *et al*., 1998; Boecker and Buerger, 2003). Due to its contractile phenotype, myoepithelial cells also express smooth muscle-specific proteins such as smooth muscle actin (SMA) and calponin (Adriance *et al*., 2005; Espinosa de los Monteros *et al*., 2002). The hunt for specific markers of the different types of epithelial cells is ongoing and several markers have already been successfully used in human and canine mammary tissues (Gama *et al*., 2003; Reis-Filho *et al*., 2003) (Fig. 1B). Some fundamental considerations will now be addressed, regarding a few selected molecular markers.

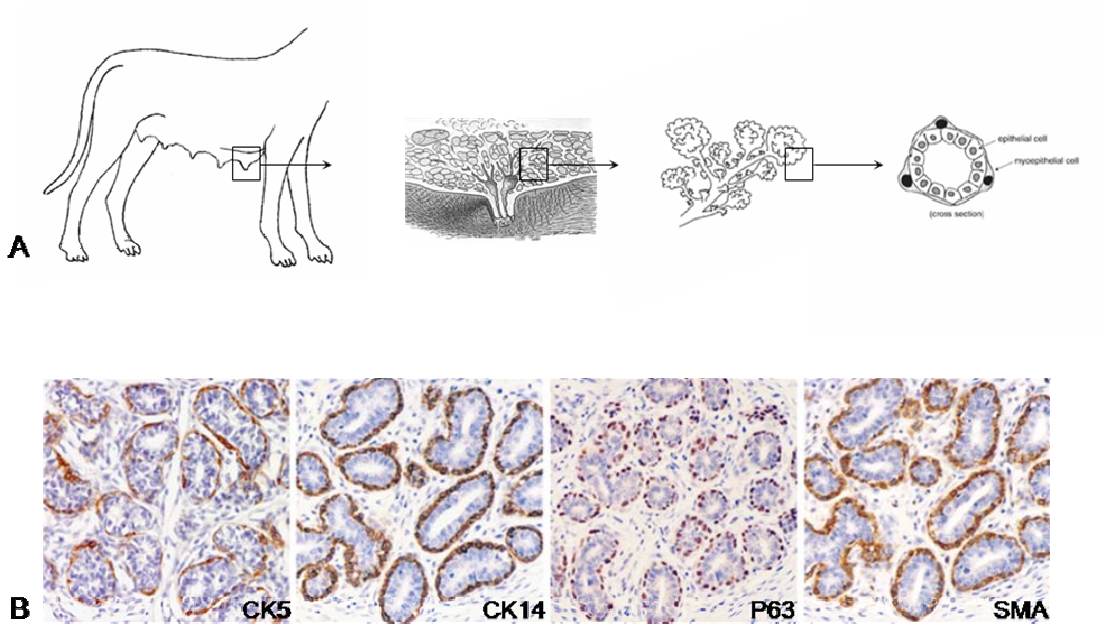

Fig. 1. Normal canine mammary gland: schematic representation (A) and immunohistochemical reactivity to basal/myoepithelial cell markers (B). A. Schematic representation of canine mammary gland, showing its anatomical location and organization. Mammary epithelium is organized as a bilayer, with a luminal layer of secretory epithelial cells, and a basal layer of myoepithelial cells. B. Normal mammary gland stained with antibodies to CK5, CK14, p63 and SMA, highlighting the basally located myoepithelial cells.

Cytokeratins (CK). CK are the typical intermediate filament proteins of epithelia and are essential for normal tissue structure and function (Schweizer *et al*., 2006). Besides major components of the epithelial cytoskeleton, CK are highly dynamic and have also been involved in intracellular signalling pathways (Moll *et al*., 2008).

The human keratin family shows an outstanding degree of molecular diversity and includes 54 distinct elements (Moll *et al*., 2008). CK are encoded by *KRT* genes mostly clustered on paralogous regions of 12q and 17q chromosome arms and are classified, either upon type and isoelectric point, i.e. type I acidic (CK9-10, CK12-28 and CK31-40) and type II neutral-basic (CK1-8 and CK71-86) or upon molecular mass, i.e. low and high molecular weight CK (such as CK18/19 and CK5/6, respectively) (Chu and Weiss, 2002; Moll *et al*., 2008).

The stability of intermediate filaments makes it possible to characterize and study tumour histogenesis but although epithelial tissues tend to retain their characteristic CK pattern throughout carcinogenesis, modulations may occur within a certain range of possibilities during carcinoma development and progression (Malzahn *et al*., 1998; Abd El-Rehim *et al*., 2004; Birnbaum *et al*., 2004; Laakso *et al*., 2005). It has long been suggested that certain constituent proteins of the cytoskeletal intermediate filaments may be of relevance with respect to the biological behaviour and prognosis of human breast carcinomas (Dairkee *et al*., 1987; Raymond and Leong, 1989; Takei *et al*., 1995; Schaller *et al*., 1996). In fact, the immunoexpression of basal-type CK, such as CK5, CK14 and CK17, has been associated with a poor prognosis for many years (Dairkee *et al*., 1987; Malzahn *et al*., 1998). After the rediscovery of basal-like carcinomas by gene expression microarray analysis, numerous immunohistochemical studies confirmed these earlier findings (van de Rijn *et al*., 2002; Abd El-Rehim *et al*., 2004; Gusterson *et al*., 2005).

A few number of studies are available concerning cytokeratin expression in canine mammary tumours (Hellmén and Lindgren, 1989; Destexhe *et al*., 1993b; Griffey *et al*., 1993); however, Griffey and coworkers (1993), based on CK14 expression, already described a basal phenotype for canine mammary carcinomas, which was also characterized by an aggressive clinical behaviour (Griffey *et al*., 1993).

Placental cadherin (P-cadherin). P-cadherin is a member of the cadherin family, along with epithelial cadherin (E-cadherin) and neural cadherin (N-cadherin). Cadherins are calcium-dependent cell-to-cell adhesion molecules which play critical roles during embryonic development and in the maintenance of normal tissue architecture (Nose and Takeichi, 1986; Nose *et al*., 1987; Takeichi, 1991, 1993, 1995; Gumbiner, 1996).

In a development setting, P-cadherin is transiently expressed in various tissues (Hirai *et al*., 1989); it was localized to the cap cells of terminal end buds in the developing murine mammary gland, which might represent mammary stem cells (Williams and Daniel, 1983; Daniel *et al*., 1995).

P-cadherin expression in adult tissues is limited to epithelium, located at cell-cell boundaries (Shimoyama *et al*., 1989; Shimoyama and Hirohashi, 1991). Unlike E-cadherin, which is broadly distributed in all epithelial tissues, P-cadherin exhibits a singular pattern of expression, co-localizing partially with E-cadherin and being restricted to the basal proliferative cell layer of the majority of stratified epithelia (reviewed by Paredes *et al*., 2007).

In normal human and canine mammary gland, P-cadherin is restricted to myoepithelial cells, representing a sensitive marker for this cell type (Rasbridge *et al*., 1993; Palacios *et al*., 1995; Kovacs and Walker, 2003; Gama *et al*., 2004). However, during lactation, P-cadherin is not found at cell-cell borders, as expected for an adhesion molecule, but rather appears to be secreted by epithelial cells (Soler *et al*., 2002; Gama *et al*., 2002).

In human breast cancer, P-cadherin was found to be expressed by a subset of carcinomas, frequently with a basal epithelial phenotype (Arnes *et al*., 2005). P-cadherin is commonly identified in medullary and metaplastic carcinomas, further suggesting a basal/myoepithelial cell histogenetic origin or line of differentiation for these tumours (Han *et al*., 1999; Reis-Filho *et al*., 2003). P-cadherin expressing tumours are usually associated with aggressive behaviour and poor outcome (Palacios *et al*., 1995; Peralta Soler *et al*., 1999; Gamallo *et al*., 2001; Kovacs *et al*., 2003; Paredes *et al*., 2002, 2005; Arnes *et al*., 2005), which has raised the interest on P-cadherin as a potential prognostic marker for breast cancer. P-cadherin expression was found inversely correlated with hormonal receptor status (Paredes *et al*., 2002a, 2002b) and it seems to be associated with an estrogen-independent tumour growth (Paredes *et al*., 2002a). Some authors actually described this molecule as an independent marker of

poor prognosis (Peralta Soler *et al*., 1999), with its expression highly predictive of a poor outcome in small, node-negative breast cancers (Arnes *et al*., 2005).
In canine mammary tumours, we have also described P-cadherin expression in a subset of malignant carcinomas. A significant association was found between its expression and tumour type, being highly positive in carcinosarcoma and spindle cell carcinoma, favouring a probable basal/myoepithelial differentiation (Gama *et al*., 2004).

P63. P63 is a recently characterized member of the p53 family (Yang *et al*., 1998; Kaelin, 1999; Little and Jochemsen, 2002). *P63* gene is located on chromosome 3q27 (Yang *et al*., 1998) and exhibits a high homology to p53, leading to the early speculation that p63 would also function as a tumour suppressor (Yang and McKeon, 2000; Westfall and Pietenpol, 2004). Despite its homology with p53, *p63* gene encodes at least six different proteins, grouped in two distinct classes: one containing a region that is similar to p53 transactivation domain (TAp63 isoforms) and another lacking this domain (ΔNp63 isoforms) (Yang *et al*., 1998). *P63* is rarely mutated (Osada *et al*., 1998; Hagiwara *et al*., 1999) and several studies described an overexpression of p63 splice variants in a subset of human epithelial tumours, sometimes associated with gene amplification, suggesting that p63 can act as an oncogene (Crook *et al*., 2000; Hibi *et al*., 2000; Park *et al*., 2000; Yamaguchi *et al*., 2000; Massion *et al*., 2003). Supporting this hypothesis, it was recently shown that deregulated TAp63 isoform predisposes to tumour development and progression (Koster *et al*., 2006) and that p63 contributes to cell invasion and migration in squamous cell carcinoma of the head and neck (Gu *et al*., 2008).
Although p63 function is not fully understood, the striking epithelial defects seen in p63-deficient mice suggest that this gene plays a key role in maintaining basal/progenitor epithelial cell populations (DiRenzo *et al*., 2002). Whereas p53-/- mice are developmentally normal but prone to neoplastic disease (Donehower *et al*., 1992), p63 knockout mice have severe developmental abnormalities. Specifically, the p63-/- mice die shortly after birth and are deficient in the development of several epithelial tissues such as skin, prostate, mammary gland, and urothelia (Mills *et al*., 1999; Yang *et al*., 1999). Recently, Koster *et al*. (2004) demonstrated that TAp63 isoforms are the first to be expressed during embryogenesis and are required for

initiation of epithelial stratification. This program further involves a shift in the balance between p63 isoforms towards ΔNp63 to allow terminal differentiation. After epidermis maturation, persistently elevated p63 levels in the basal layer are required for the maintenance of the basal cell proliferative potential (Koster *et al*., 2004).

Immunohistochemical analyses show p63 protein localization and expression in the basal/progenitor cells of several adult epithelial tissues such as the epidermis, hair follicles, sweat glands, cervix, tongue, esophagus, mammary glands, prostate, and urogenital tract, with ΔNp63α being the predominant, if not only, p63 variant expressed (Yang *et al*., 1998; Parsa *et al*., 1999; Signoretti *et al*., 2000; Barbareschi *et al*., 2001; Pellegrini *et al*., 2001; Di Como *et al*., 2002; Westfall *et al*., 2003).

P63 is consistently expressed in basal/myoepithelial cells of normal human and canine mammary gland and in tumours with basal/myoepithelial cell features (Barbareschi *et al*., 2001; Nylander *et al*., 2002; Wang *et al*., 2002; Gama *et al*., 2003; Reis-Filho and Schmitt, 2003; Ribeiro-Silva *et al*., 2003b; Ramalho *et al*., 2006). Thus, p63 has been proposed as a reliable basal/myoepithelial cell marker, being expressed by basal-like breast carcinomas, including metaplastic type (Reis-Filho *et al*., 2003; Laakso *et al*., 2005; Matos *et al*., 2005). Some human breast cancer studies found an association between its expression and high grade, large tumour size, nodal metastasis and ER negativity (Ribeiro-Silva *et al*., 2003a).

Prognostic markers in mammary cancer

Recently, prognostic value has been claimed for several molecular variables in canine mammary cancer studies (Zaidan Dagli, 2008), including cell proliferation markers (Peña *et al*., 1998; Sarli *et al*., 2002; De Matos *et al*., 2006), receptor proteins (Graham *et al*., 1999; Geraldes *et al*., 2000; Nieto *et al*., 2000), oncogenes/tumour suppressor genes (Ahern *et al*., 1996; Lee *et al*., 2004) and adhesion molecules (Brunetti *et al*., 2005; Matos *et al*., 2006), among others (Queiroga *et al.*, 2005; Pinho *et al.*, 2007). Despite their recognition as relevant prognostic indicators in human breast cancer, some of these factors have generated contradictory results and still lack validation in the veterinary area.

Tumour cell proliferation. Proliferation is a key feature in tumour progression and has been extensively investigated to evaluate prognosis in canine mammary cancer (Destexhe *et al*., 1993a; Bratulic *et al*., 1996; Peña *et al*., 1998; Sarli *et al*., 2002; Zuccari *et al*., 2004; De Matos *et al*., 2006). Several methods were performed including AgNOR quantification (Bostock *et al*., 1992; Bratulic *et al*., 1996; Lohr *et al*., 1997), DNA flow cytometry measurement of S-phase fraction (Hellmén *et al*., 1993) and immunohistochemical analysis of PCNA and Ki-67 (Preziosi *et al*., 1995; Peña *et al*., 1998; Geraldes *et al*., 2000; Sarli *et al*., 2002; Zacchetti *et al*., 2003; De Matos *et al*., 2006).

Currently, proliferation is widely estimated by immunohistochemical assessment of Ki-67, both in human (Bouzubar *et al*., 1989; Brown and Gatter, 1990; Veronese and Gambacorta, 1992; Barginear *et al*., 2008) and in canine cancers (Peña *et al*., 1998; Sarli *et al*., 2002; De Matos *et al*., 2006), with most studies describing an association between high proliferation and poor prognosis. Ki-67 is a non-histone nuclear protein detected in all cell cycle phases except the resting phase (G0) and is therefore a direct indicator of the tumour growth fraction (Durchow *et al*., 1994).

Hormone receptors. Oestrogen and oestrogen receptors (ER) play essential roles in both normal breast development and breast cancer progression (Pearce and Jordan, 2004). Oestrogen exerts its biological effects usually by binding to ER (ERα and ERβ), which mainly exists in the nucleus as a member of the nuclear receptor superfamily of transcription factors. The oestrogen–ER complex through genomic and nongenomic pathways, leads to nuclear and extranuclear processes that promote cellular proliferation and differentiation (Fig. 2) (Murphy and Watson, 2002; Yamaguchi, 2007). Growth factor signalling pathways can also activate ER via phosphorylation, in a ligand-independent manner (Le Goff *et al*., 1994; Lee *et al*., 2001).

Progesterone Receptor (PR) is an oestrogen-regulated gene and its expression is therefore thought to indicate a functioning ER pathway. Theoretically, the assessment of PR should assist in predicting response to hormonal therapy more accurately. In keeping with this, there is some evidence that tumours positive for PR are more likely to respond to tamoxifen (Ravdin *et al*., 1992; Bardou *et al*., 2003) but the predictive value of PR positivity in the absence of ER is still controversial (Payne *et al*., 2008).

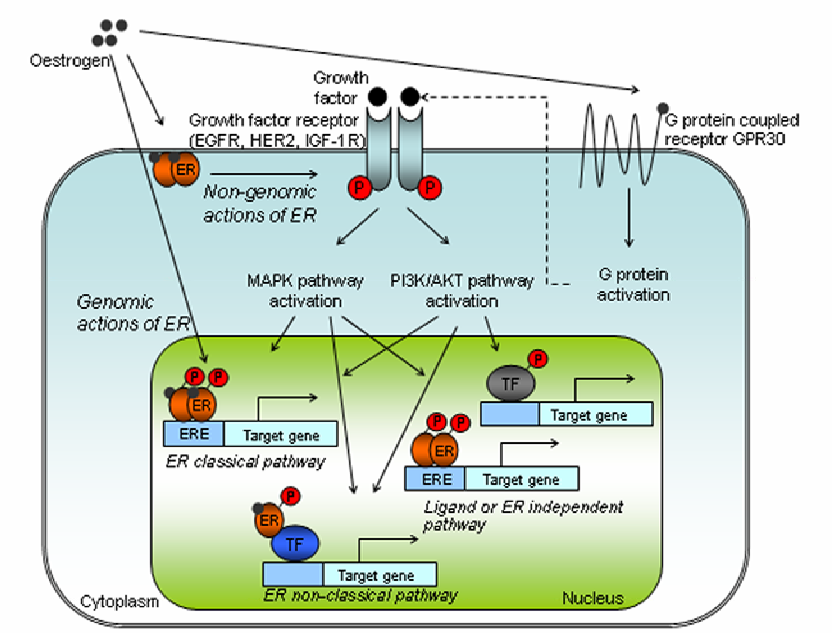

Fig. 2. Simplified overview of intracellular estrogen action mechanisms. In the classical genomic pathway, ligand-bound ERs bind directly to the estrogen response element (ERE) present in the target gene promoters. Alternatively, in the non-classical pathway ER acts as a coactivator via interaction with other transcription factors (TF), which regulates the gene transcriptions at their specific DNA sites. Membrane-initiated (non-genomic) steroid signalling has also been reported, either, controversially, as a small pool of ER within the plasma membrane, or via non-ER proteins, such as GPR30. The former can contribute to the oestrogenic response via cross-talk with growth factor-mediated pathways, which activate targeted transcription factors and/or coactivators via phosphorylation, in an ER dependent or independent manner. The latter activates G-protein signalling pathways, which result in cleavage and release of membrane-bound growth factors such as EGF, which activates its receptor and initiates intracellular kinase cascades (adapted from Yamaguchi, 2007 and Speirs and Walker, 2007).

In humans, it has been standard practice for 25 years to analyze all invasive breast cancers for hormone receptor content as a means of estimating prognosis and predicting responsiveness to endocrine treatment (Yeh and Mies, 2008). The majority of available data in canine literature concerning steroid receptors are based on biochemical assays (Mialot *et al.*, 1982; Martin *et al.*, 1984; Parodi *et al.*, 1984;

Rutteman *et al*., 1988b; Sartin *et al*., 1992; Donnay *et al*., 1993). In human breast cancer studies, these techniques were replaced by immunohistochemical methods after the development of reliable monoclonal antibodies against oestrogen and progesterone receptors (Allred *et al*., 1990). Recent studies performed on canine tissues have proven steroid receptors value in characterizing subgroups with different prognosis among female dogs with mammary cancer (Graham *et al*., 1999; Nieto *et al*., 2000). Despite some studies describing an association between the absence of ER/PR or both and shorter survival time (Martin *et al*., 1984; Sartin *et al*., 1992; Nieto *et al*., 2000; Martin de las Mulas, 2005), others failed to find such a correlation (Millanta *et al*., 2005). Thus, there is still insufficient data in the literature on the prognostic significance of hormonal status and its application into diagnostic routine remains a matter of debate.
ER inhibition through endocrine targeting, either directly using weak oestrogen agonists (Selective Oestrogen Receptor Modulators) or indirectly by blocking the conversion of androgens to oestrogen (e.g. aromatase inhibitors), forms the mainstay of adjuvant and metastatic human breast cancer therapy (Payne *et al*., 2008). In dogs, there is no convincing data indicating that hormonal treatment improves prognosis. In fact, although ER-positive canine mammary tumour cell lines have been shown to respond to the selective ER modulator tamoxifen (Sartin *et al*., 1993), adjuvant endocrine therapy is not currently used. Treatment of bitches with tamoxifen has been reported to produce estrogen-like side effects and at present is not advised for dogs (Rutteman *et al*., 2001).

Human Epidermal Growth Factor Receptor-2 (HER-2). The HER family of receptor tyrosine kinases includes four closely related members: Epidermal Growth Factor Receptor (EGFR, also called HER-1 or c-erbB-1), HER-2 (also called c-erbB-2 or neu), HER-3 (also called c-erbB-3), and HER-4 (also called c-erbB-4) (Holbro and Hynes, 2004). These receptors share a common structure comprising an extracellular ligand-binding domain, a transmembrane domain and an intracellular domain with tyrosine kinase activity. Binding of growth factor ligands causes homo- or hetero-dimerization with another family member, which leads to receptor-linked tyrosine kinase activation. This activation triggers a network of intracellular signalling pathways, mainly the mitogen activated protein kinase (MAPK) and the

phosphatidylinositol 3 kinase (PI3K)-AKT pathways, which produce diverse cellular events including cell proliferation, adhesion, migration, differentiation, angiogenesis and inhibition of apoptosis (Fig. 3) (Hanna *et al*., 1999; Yarden and Sliwkowski, 2001; Kumar and Wang, 2002).

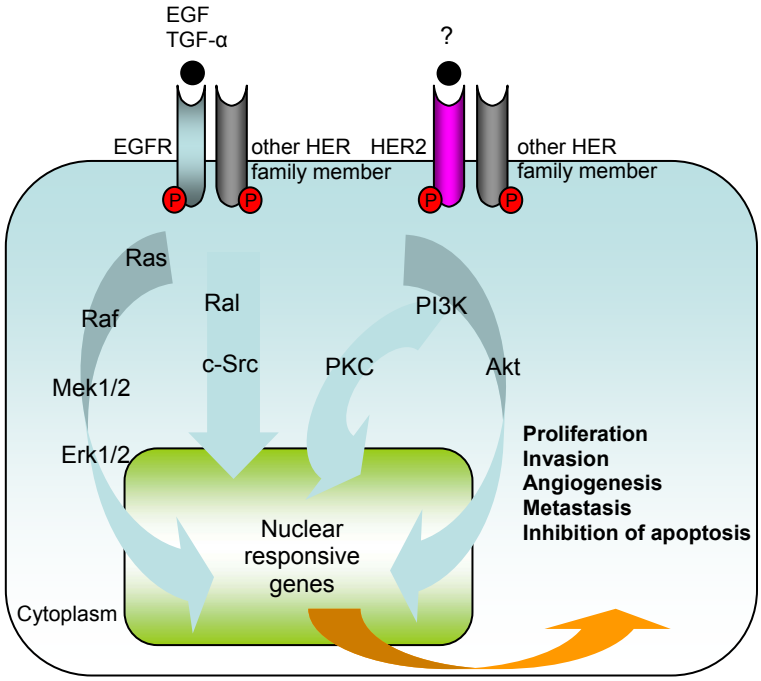

Fig. 3. Signalling pathways of HER family members. Ligand binding induces receptor dimerization and subsequent autophosphorylation of distinct tyrosine residues, creating docking sites for adaptor molecules and leading to the activation of downstream effector molecules. A variety of signalling pathways results in pleiotropic effects, including cell proliferation, control of the cell cycle, regulation of apoptosis and survival, and alterations in cell migration and invasiveness (adapted from Prenzel *et al*., 2001 and Vlahovic and Crawford, 2003).

The human *HER2* gene maps to chromosome 17q21 and encodes a 185 kDa glycoprotein. It is reported to be amplified and overexpressed in several types of human tumours, including breast cancer (Hynes and Lane, 2005). HER-2 overexpression is found in 15–30% of human breast carcinomas and correlates with more aggressive clinicopathological features, drug resistance or sensitivity to specific chemotherapy and hormonal therapy regimens in breast cancer (Slamon *et al*., 1987; Revillion *et al*., 1998; Yamauchi *et al*., 2001; Burstein, 2005). Amplification is the

predominant mechanism of gene overexpression and is present in about 85–90% of the cases (Hoang *et al*., 2000; Jimenez *et al*., 2000).

HER-2 was one of the first oncogenes studied in clinical samples of invasive breast cancer. Its early significance as a prognostic factor has been surpassed by its key importance as a predictive factor of response to particular systemic therapies, notably trastuzumab (Herceptin™), a humanized monoclonal antibody which has been shown in several studies to improve response rates, time to progression and overall survival (Slamon *et al*., 2001; Romond *et al*., 2005; Smith *et al*., 2007).

While the expression of HER-2 has been extensively investigated in human breast tumours, only a limited number of studies are available concerning HER-2 status in canine tumours (Ahern *et al*., 1996; Schafer *et al*., 1998; Rungsipipat *et al*., 1999; Matsuyama *et al*., 2001; Martin de las Mulas *et al*., 2003). Furthermore, and despite HER-2 recognition as a prognostic factor in human breast cancer (Slamon *et al*., 1987; Revillion *et al*., 1998), the significance of HER-2 overexpression in dogs with mammary carcinoma is still unclear. Some studies have shown that either HER-2 amplification (Ahern *et al*., 1996) or protein overexpression (Rungsipipat *et al*., 1999) are present in canine mammary carcinomas; nevertheless, a subsequent study addressing simultaneously HER-2 protein and gene status found no gene amplification in overexpressing tumours (Martin de las Mulas *et al*., 2003).

Canine *HER-2* has been mapped to 1q13.1 and cytogenetic studies of canine tumours revealed that this region is very often affected by clonal chromosome aberrations, which might be associated with HER-2 protein overexpression (Murua Escobar *et al*., 2001). In canine mammary carcinomas, HER-2 overexpression was found usually associated with established indicators of poor prognosis (Martin de las Mulas *et al*., 2003; Dutra *et al*., 2004). However, a recent study performed by Hsu *et al*. (2007) revealed that HER-2 overexpression in canine malignant mammary tumours is associated with higher survival rates (Hsu *et al*., 2007).

Epidermal Growth Factor Receptor (EGFR). The *EGFR* gene maps to human chromosome 7p11.2-p2 and encodes for a 170 kDa transmembrane tyrosine kinase which is activated by several ligands, including EGF and TGF-α (reviewed in Suo and Nesland, 2002). EGFR was the first tyrosine kinase transmembrane receptor to be

directly linked with human cancer (Hynes and Lane, 2005). Its expression in normal and neoplastic breast has been extensively studied, since EGFR is required for normal mammary development and lactation (Kumar and Wang, 2002). Recent studies have showed EGFR to be frequently expressed in basal cell layers and in myoepithelial cells (Santini *et al*., 2002; DiRenzo *et al*., 2002) and several authors pointed out EGFR as a possible basal cell marker (Korsching *et al*., 2002; Nielsen *et al*., 2004).

EGFR overexpression is observed in approximately 16-48% of all breast cancers, although methodology and positivity criteria differ widely among studies (Klijn *et al*., 1992; Fox *et al*., 1994; Toi *et al*., 1994; Tsutsui *et al*., 2002; Rampaul *et al*., 2005). EGFR expression is common in basal-like breast cancers, being found in up to 60% of basal-like breast carcinomas (Nielsen *et al*., 2004; Reis-Filho *et al*., 2005, 2006; Livasy *et al*., 2006; Turner and Reis-Filho, 2006).

EGFR overexpression has been shown to be associated with aggressive biological properties and poor clinical outcome (Nicholson *et al*., 1990; Tsutsui *et al*., 2002; Tovey *et al*., 2004), but the validity of EGFR as a useful prognostic factor for human breast cancer is still uncertain (Nicholson *et al*., 2001; Rampaul *et al*., 2004; Park *et al*., 2007). The interest in EGFR is further enhanced by the availability and recent FDA approval of specific EGFR tyrosine kinase inhibitors (Bhargava *et al*., 2005).

Molecular mechanisms for EGFR overexpression in the majority of breast cancer cases are yet to be identified (Reis-Filho *et al*., 2006). *EGFR* gene amplifications are rather uncommon in breast cancer, with the exception of basal-like carcinomas (Reis-Filho *et al*., 2005, 2006; Park *et al*., 2007). In addition, *EGFR* activating mutations represent remarkably rare findings (Bhargava *et al*., 2005; Weber *et al*., 2005; Reis-Filho *et al*., 2006) and other regulatory mechanisms are certainly involved, possibly at the transcriptional level (Kersting *et al*., 2004, 2006; Park *et al*., 2007; Sassen *et al*., 2008).

In contrast to human literature (Klijn *et al*., 1992; Bhargava *et al*., 2005; Reis Filho *et al*., 2005, 2006; Park *et al*., 2007), a limited number of reports are available concerning EGFR status in canine mammary tissues, with most studies based on biochemical assays (Nerurkar *et al*., 1987; Rutteman *et al*., 1990; Donnay *et al*., 1993; Rutteman *et al*., 1994; Donnay *et al*., 1996). These studies failed to find a relation between EGFR concentrations and clinicopathological parameters. Yet, some studies found an inverse correlation between EGFR and ER concentrations in malignant tumours (Nerurkar *et al*., 1987), while others described a positive (Donnay *et al*., 1993; Donnay *et al*., 1996)

or no existing correlation (Rutteman *et al*., 1994). EGFR mRNA expression was recently described in canine mammary tissues, with controversial results in normal mammary gland (Matsuyama *et al*., 2001). According to this study, EGFR expression was present in tumours but it was not observed in normal tissues, contradicting previous canine (Rutteman *et al*., 1994) and human findings (Moller *et al*., 1989; Santini *et al*., 2002).

E-Cadherin. E-cadherin (also called uvomorulin, L-Cam, cell-Cam120/80 or Arc-1) is a 120 kDa transmembrane glycoprotein whose extracellular domain promotes cell-to-cell adhesion, while the intracellular domain interacts with catenins (α-, β- and γ-catenins) which link cadherins to the actin cytoskeleton (Fig. 4) (Ozawa *et al*., 1989; Vleminckx *et al*., 1991; Knudsen *et al*., 1998). β-catenin also participates in a signal transduction cascade as part of the Wnt signalling pathway (Berx *et al*., 2001; Brown, 2001). Another catenin-like molecule, p120, has been identified in association with E-cadherin at the cell-cell junctions, although this complex does not appear to form a link with the actin cytoskeleton (Reynolds *et al*., 1994).

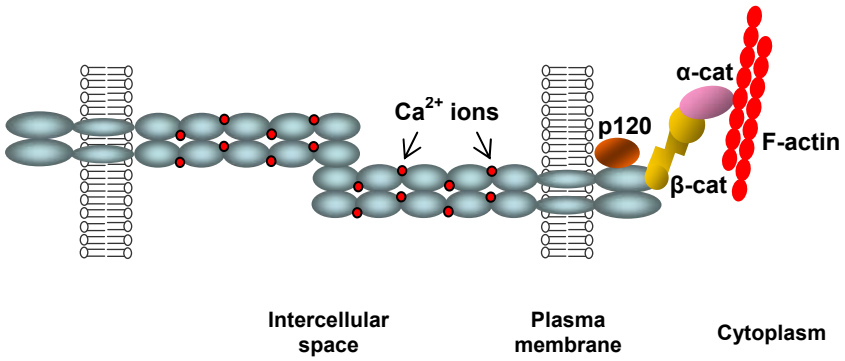

Fig. 4. Schematic representation of the classical cadherin-catenin complex. Classical cadherins (blue), which mediate calcium-dependent (red) intercellular adhesion, are composed by an extracellular domain, a transmembrane domain and a cytoplasmic domain. This last domain comprises a juxtamembrane domain, which binds p120-catenin (orange), and a catenin-binding domain, which binds β-catenin (yellow). β-catenin binds α-catenin (violet), which establishes a direct link between the cadherin-catenin complex and the actin cytoskeleton (red) (adapted from Paredes *et al*., 2007).

Normal E-cadherin expression and function are essential for the induction and maintenance of a polarized and differentiated epithelium during embryonic development (Takeichi, 1991). In adult epithelial tissues, an intact complex is required for the maintenance of normal intercellular adhesion. In the light of this, several authors have proposed that E-cadherin might function as an invasion suppressor molecule such that a disturbed function of E-cadherin-catenin complex theorethically enhances the tumour cell invasive potential (Wijnhoven *et al*., 2000).

In normal human and canine mammary gland, E-cadherin is expressed by both luminal and myoepithelial cells at cell-cell borders (Rasbridge *et al*., 1993; Palacios *et al*., 1995; Restucci *et al*., 1997). In human breast cancer, the loss or reduction of the E-cadherin-catenin complex has been extensively associated with tumour progression (Oka *et al.*, 1993; Siitonen *et al.*, 1996; Bukholm *et al*., 1998; Heimann *et al.*, 2000; Madhavan *et al.*, 2001). In general, loss of E-cadherin expression correlates with undifferentiated breast carcinomas, but the available studies differ with regard to its association with survival and its value as a prognostic marker is still controversial (Knudsen and Wheelock, 2005; Gould Rothberg and Bracken, 2006). Some studies report that the combination of E-cadherin and one of the catenins is of better prognostic value than the evaluation of individual components (Zschiesche *et al.*, 1997; Gofuku *et al*., 1999).

The role of E-cadherin and catenins in canine mammary tumours is still poorly understood (Restucci *et al*., 1997; Reis *et al*., 2003; Sarli *et al.*, 2004; Brunetti *et al*., 2005; Matos *et al*., 2006; de Matos *et al*., 2007; Nowak *et al*., 2007; Rodo and Malicka, 2008). Brunetti *et al*. (2005) reported that reduced E-cadherin/β-catenin expression was associated with invasion, but no correlation was found regarding survival. More recent studies also described a significant correlation between E-cadherin loss and several classic prognostic features (Matos *et al*. 2006), as well as with invasion and lymph node metastases, suggesting this molecule as a potential prognostic marker for canine mammary cancer (de Matos *et al*., 2007).

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3. AIMS AND OUTLINE OF THE THESIS

With the purpose of better elucidate canine mammary gland tumour biopathology, we have intended to provide new insights on their histogenesis/differentiation, prognosis and molecular classification. To accomplish this goal, we defined the following specific aims:

. To perform an extensive clinicopathological characterization of canine mammary benign and malignant tumours. To study possible associations between host and tumour characteristics and biologic behaviour of canine mammary tumours.

. To evaluate the immunohistochemical expression of the cell adhesion molecules E-cadherin, P-cadherin and β-catenin in a series of canine malignant mammary tumours and their relation to clinicopathological parameters, proliferation and survival.

. To evaluate the immunohistochemical expression of EGFR in a series of benign and malignant canine mammary tumours. To evaluate its expression in relation to clinicopathological parameters and survival.

. To evaluate the immunohistochemical expression of the luminal cell marker CK 19 and basal/myoepithelial cell markers (CK5, CK14, p63, calponin, smooth muscle actin and P-cadherin) in a series of canine malignant mammary tumours. To study CK19 prognostic significance and its relationship with clinicopathological parameters and basal/myoepithelial cell markers expression.

. To identify molecular phenotypes in a series of canine mammary carcinomas based on the application of a human classification scheme, by using a surrogate panel of immunohistochemical markers (ER, HER-2, CK5, P63 and P-cadherin). To explore the relationship of these distinct phenotypes with clinicopathological parameters and survival.

Chapter II

Canine mammary gland tumours: clinical and pathological parameters as predictors of overall and disease-free survival - a univariate and multivariate analysis

Gama A, Alves A, Schmitt F (submitted)

Canine mammary gland tumours: clinical and pathological parameters as predictors of overall and disease-free survival - a univariate and multivariate analysis

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Abstract

A hundred and fifty six canine mammary tumour specimens (46 benign and 110 malignant) were clinically and histopathologically characterized. In order to investigate the prognostic value of clinical and pathological variables, a follow-up study was performed in 69 female dogs for a minimum period of 12 months after surgical procedure. Univariate analysis showed that tumour size, histological type, tumour growth, differentiation grade, stromal and lymphovascular invasion, lymph node status, mitotic and Ki-67 labelling indices were significantly associated with overall and disease-free survival. Skin ulceration was only associated with poorer overall survival rate. Cox regression multivariate analysis revealed lymph node status as the only independent prognostic factor.

Keywords: canine; mammary tumour; prognosis

Introduction

Mammary tumours are the most common neoplasias in female dogs, representing a serious problem worldwide (Misdorp *et al*., 1999; Zaidan Dagli, 2008). Malignant tumours account for up to 50% of mammary neoplasms and the search for prognostic markers has been increasing in the last decades, in order to better estimate the individual

risk of unfavourable clinical outcome (Misdorp, 2002; Sorenmo, 2003; Zaidan Dagli, 2008).

Although some clinicopathological factors have been recognized by several studies as reliable prognostic factors, a number of discrepancies and controversial results still exist concerning this subject. Tumour size (Bostock, 1975; Misdorp and Hart, 1976; Yamagami *et al*., 1996b; Chang *et al*., 2005; Martin de las Mulas *et al*., 2005), skin ulceration (Hellmén *et al*., 1993; Peña *et al*., 1998; Queiroga and Lopes, 2002), tumour type (Misdorp and Hart, 1976; Hellmén *et al*., 1993; Chang *et al*., 2005) and grade (Karayannopoulou *et al*., 2005; Martin de las Mulas *et al*., 2005) and presence of lymph node metastasis (Hellmén *et al*., 1993; Yamagami *et al*., 1996b; Queiroga and Lopes, 2002; Philibert *et al*., 2003; Chang *et al*., 2005) have been considered as good prognosticators by many investigators. However, clinical features such as animal age and breed, ovariohysterectomy status and tumour location (Schneider *et al*., 1969; Misdorp and Hart, 1976; Hellmén *et al*., 1993; Yamagami *et al*., 1996a, 1996b; Peña *et al*., 1998; Sorenmo *et al*., 2000; Rutteman *et al*., 2001; Queiroga and Lopes, 2002; Philibert *et al*., 2003; Chang *et al*., 2005) usually generate more controversial results.

In the present study, we have characterized a series of benign and malignant canine mammary tumours, both at clinical and pathological level. Clinicopathological parameters were compared between benign and malignant tumours and a survival study was performed in the malignant group, in order to investigate the possible association of these clinicopathological features and clinical outcome.

Material and methods

Tumour specimens

Canine mammary gland tumour specimens were obtained from the archives of the Histopathology Laboratories of the University of Trás-os-Montes and Alto Douro, Vila Real and from the Institute of Biomedical Science at the University of Porto. Tumour samples were surgically removed from 153 female dogs by lumpectomy or mastectomy (regional or radical) in private clinical practices (the majority from the Northern region of Portugal) and in the hospitals of the above mentioned institutions. From the available archival material obtained between 1999 and 2007, selected benign (n=46) and

malignant (n=110) mammary tumours were studied. The material had been fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (3 μm) were cut and stained with haematoxylin and eosin (HE) for histological examination.

Clinicopathological parameters evaluation

Clinical gathered data included animal breed, age, reproductive status (intact; ovariohysterectomized prior to tumour development; ovariohysterectomized with mastectomy), previous administration of oestrous-prevention medications and tumour characteristics (location, size, skin ulceration). Tumour size was defined as the maximum diameter and tumours were grouped according to the TNM WHO staging of canine mammary tumours (Rutteman *et al*., 2001) in: tumours with less than 3 cm; tumours with 3-5 cm and tumours larger than 5 cm.

All tumour samples were revised and reclassified independently by three observers from haematoxylin and eosin (HE) stained sections, according to the World Health Organization (WHO) criteria for canine mammary neoplasms (Misdorp *et al*., 1999). Other histopathological parameters evaluated included: intra-tumoural necrosis (presence vs. absence), mode of growth (expansive vs. infiltrative), characterization of inflammatory cellular infiltrates (infiltrate type and extent), stromal/lymphovascular invasion (presence vs. absence) and lymph node metastases (presence vs. absence).

Histological grade was evaluated in malignant epithelial neoplasms, according to the Nottingham method for human breast tumours (Elston and Ellis, 1998), which is based on the assessment of three morphological features: tubule formation, nuclear pleomorphism and mitotic counts. Each of these features was scored as 1, 2 or 3 to indicate whether it was present in slight, moderate or marked degree, respectively, giving a putative total of 3-9 points. Grade was allocated by an arbitrary division of the total points as follows: grade I (well differentiated), 3, 4 or 5 points; grade II (moderately differentiated), 6 or 7 points; and grade III (poorly differentiated), 8 or 9 points. Mitotic counts were assessed as the number of mitoses per 10 high power fields (40x) at the tumour periphery, by using a Nikon Labophot microscope (area=0,152 mm^2).

Proliferation indices

For Ki-67 immunostaining, a monoclonal antibody was used (MIB-1, 1:50, Dakocytomation) and the immunohistochemical technique was performed according to the streptavidin-biotin-peroxidase complex (ABC) method. Briefly, tissue sections were deparaffinized, rehydrated and antigen retrieval was carried out. Slides were incubated with 0.2 mg/mL trypsin (Merck) in phosphate buffered saline (PBS) for 10 min at 37 ºC prior to microwave treatment (3 x 5 min) in a 10 mM citrate buffer, pH 6.0. After cooling 20 minutes at room temperature, the sections were immersed in 3% hydrogen peroxide (H_2O_2) and distillated water during 30 minutes to block endogenous peroxidase activity. Non-specific staining was eliminated by 5-minute incubation with Ultra V Block (Lab Vision). Excess serum was removed, replaced by the primary antibody, and the slides were incubated overnight in a humid chamber at 4ºC. After incubation, the slides were washed and sections were incubated with biotinylated goat anti-polyvalent (Lab Vision) for 10 minutes followed by streptavidin peroxidase for 10 min (Lab Vision). Sections were rinsed thoroughly with PBS between each step of the procedure. Subsequently, the color was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) with H_2O_2 in PBS buffer for 10 minutes. Slides were counterstained with Gill's hematoxylin, dehydrated, and mounted. Adjacent normal mammary tissues were used as internal positive controls. Negative controls were carried out by replacing the primary antibody with PBS.

Ki-67 immunostaining was nuclear and considered positive regardless of the intensity. Mitotic and Ki-67 labelling indices were determined both on benign and malignant lesions, by counting 1,000 neoplastic cells in the most mitotically active areas, at high magnification (40x), with the help of a microscopic grid (Zeiss®). Mitotic and Ki-67 indices were calculated as the percentage of tumour cells that exhibited mitotic figures or had positive staining for Ki-67, respectively.

Follow up study

After the surgical procedure, dogs presenting malignant mammary tumours were submitted to a minimum follow-up period of 12 months (range 5-74 months). Follow-up was performed by the referring surgeons and it was possible in sixty nine malignant tumour cases. The remaining cases were excluded from follow-up due to a number of

reasons: dogs died immediately after surgery, others failed clinical examinations and some ancient cases just didn't have medical records anymore. Overall survival (OS) was defined as the period between surgery and animal natural death or euthanasia due to cancer. Disease-free survival (DFS) was defined as the period of time between surgery and recurrent or metastatic disease. One dog died due to causes unrelated to the mammary tumour and it was censored at the time of death (19 months).

Statistical Analysis

For statistical analysis, categorical variable studies were performed by using the chi-square test and Fisher's exact test (two-sided). Continuous variables (mitotic and Ki-67 indices) were evaluated with the Mann-Whitney or Kruskall Wallis test.

In order to determine the effect of studied clinicopathological variables on prognosis, survival curves were generated by the Kaplan-Meier method and the survival rates compared using the log-rank test. To analyze the effect of proliferation on survival, mitosis and Ki-67 labelling indices were dichotomized to low (minor than the median value) and high groups (≥ the median value).

The Cox proportional hazard model for multivariate analysis was performed to determine the effects of different co-variables on overall and disease-free survival. Variables that were found to be important in the Kaplan-Meier analyses were included in the multivariate analysis. The hazard ratios were estimated with their 95% confidence interval. All statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, Chicago, USA), 11.5 version. $P<0.05$ was considered statistically significant.

Results

Clinical characterization

The overall clinical characteristics of our tumour series are displayed in Table 1.

Clinical information regarding animal age and breed was possible in 145 and 146 cases, respectively. The mean age of dogs at the time of surgical removal of tumours was 9.3 ± 2.5 years (range 2–16 years of age). For statistical purposes, age groups were established based on the mean age of dogs, with animals divided in young (≤9 years)

and old (>9 years) dogs. Concerning animal breed, most affected bitches were mixed breed (n=48, 33.1%), followed by Poodles (n=25, 17.2%), Cocker spaniels (n=17, 11.7%), Boxers (n=9, 6.2%) and Labrador retrievers (n=6, 4.1%). For statistical purposes, we subdivided this variable in mixed breeds (n=48), poodles (n=25), cocker spaniels (n=17) and other breeds (n=48).

Table 1. Frequencies observed in the present series for clinical parameters.

Clinical parameter	Frequencies n (%)
Age (n=146)	
≤9 years	74 (50.7%)
>9 years	72 (49.3%)
Breed (n=145)	
Mixed breed	48 (33.1%)
Poodle	25 (17.2%)
Cocker spaniel	17 (11.7%)
Others	55 (37.9%)
Ovariohysterectomy (n=98)	
No	70 (71.4%)
Yes, prior to tumour development	13 (13.3%)
Yes, performed with mastectomy	15 (15.3%)
Contraception (n=98)	
No	72 (90%)
Yes	8 (10%)
Tumour size (n=149)	
<3 cm	80 (53.7%)
3-5 cm	41 (27.5%)
>5 cm	28 (18.8%)
Tumour location (n=89)	
Cranial glands (thoracic)	8 (9%)
Medial gland (cranial abdominal)	14 (15.7%)
Caudal glands (caudal abdominal and inguinal)	50 (56.2%)
Multiple	17 (19.1%)
Skin ulceration (n=154)	
No	132 (85.7%)
Yes	22 (14.3%)

Regarding reproductive status, from 98 cases with available clinical information, 13 dogs (13.3%) have been spayed prior to tumour development, whereas in 15 (15.3%) cases ovariohysterectomy was performed with mastectomy.

Out of 80 cases, 8 dogs (10%) have been previously medicated with contraceptives. All of them developed malignant mammary tumours, but no association was found between contraception and the parameters evaluated in our study. As for tumour size, it was obtained in 149 cases. The mean maximum tumour diameter was 3.38 ± 3.24cm, with tumours ranging from 0.4 to 18cm. With regard to location, 89 cases had available information, with most tumours located in caudal abdominal and inguinal mammary glands (n=50, 56.2%) and in the right mammary chain (n=46, 51.7%). Thoracic mammary glands were involved only in 8 (9%) cases. Seventeen (19.1%) tumours presented a multiple location, usually affecting two mammary glands (n=15). As for skin ulceration, it was found in 22 (14.3%) out of 154 cases, always associated with malignancy (Fig.1).

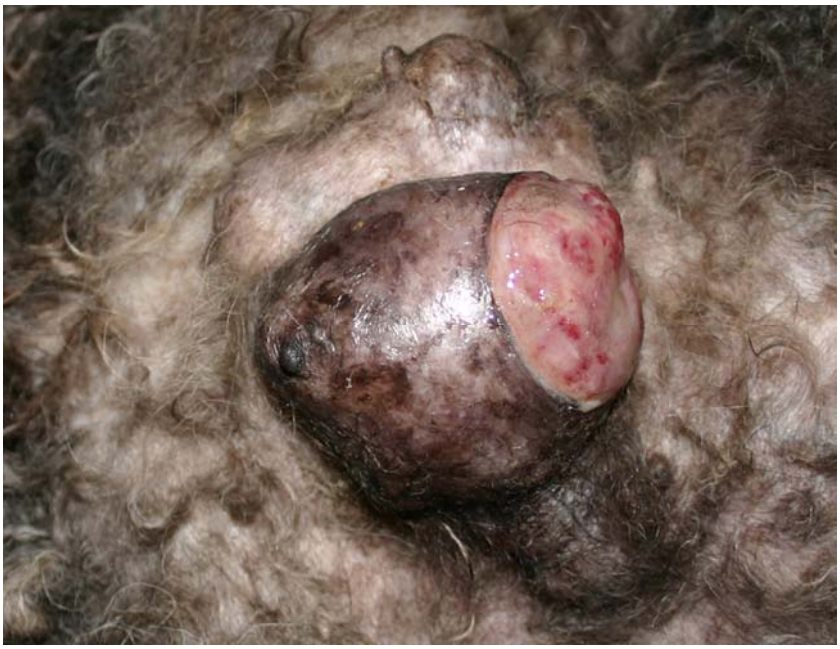

Fig. 1. Canine mammary gland tumour exhibiting extensive skin ulceration.

Histopathological characterization

The histopathological characteristics of our series are presented in Table 2. Mammary gland tumours under study were subdivided in benign and malignant tumours, being classified according to the WHO criteria for canine mammary tumours. Next, we will provide a description of the histological tumour types observed in our series.

Benign tumours (n=46). Benign mixed tumours (n=22) were characterized by the proliferation of both glandular (luminal and myoepithelial) and mesenchymal elements like cartilage (n=13) or cartilage and bone (n=9) (Fig. 2). Distinct myoepithelial cell morphologies were observed, from spindle- to stellate-cells, located in a suprabasal or interstitial position. Complex adenomas (n=13) were composed of a mixed proliferation of luminal epithelial cells and cells resembling myoepithelial cells (Fig. 3). Distinct myoepithelial cell morphologies were observed, similarly to the ones described for benign mixed tumours. Basaloid adenomas (n=11) were characterized by the proliferation of uniform cords or clusters of monomorphic basaloid epithelial cells. Centrally located cells showed squamous differentiation in the majority of cases (n=10) (Fig. 4).

Malignant tumours (n=110). Complex carcinomas (n=31) were characterized by the dual proliferation of luminal epithelial and myoepithelial cells. These tumours were usually solid, with myoepithelial cell proliferations admixed with a solid or tubulopapillary proliferation of luminal cells (Fig. 5). Most tumours were well circumscribed, frequently showing moderate cellular atypia and an expansive tumour growth. Solid carcinomas (n=27) were characterized by the arrangement of epithelial tumour cells in solid sheets, cords or nests, usually associated with a scant stromal component. These solid proliferations showed marked cellular pleomorfism and high number of mitotic figures (Fig. 6). Tubulopapillary carcinomas (n=19) were composed of a proliferation of cells resembling luminal epithelial cells showing a tubular and/or papillary arrangement (Fig. 7). Yet, the use of myoepithelial cell markers confirmed an associated myoepithelial cell component in 8 cases, not easily identified on routine diagnosis. In addition, 3 cases displayed more than 50% of a micropapillary pattern, which defines a micropapillary carcinoma in human breast tumour classification. These tumours were characterized by papillary cell clusters surrounded by empty lacunar spaces (Fig. 8). Papillae lacked a true fibrovascular core and were lined by polygonal cells showing intermediate to high grade nuclei. Carcinosarcomas (n=16) were composed of both epithelial and mesenchymal malignant components (Fig. 9), characterized by marked cellular pleomorfism, abundant mitotic figures and infiltrative tumour growth. Carcinoma in benign tumour cases (n=7) were characterized by a benign proliferation of

a dual epithelial cell population, which presented large areas of malignant epithelial proliferation, associated with moderate or marked cellular pleomorphism, sometimes exhibiting metaplastic changes. Spindle cell carcinomas (n=5) were characterized by the proliferation of infiltrative spindle cells usually arranged in solid epithelial patterns, with the formation of bundles and nests (Fig. 10). All of these tumours were positive for at least one myoepithelial cell marker.

Anaplastic carcinomas (n=3) were characterized by the proliferation of pleomorphic epithelial cells, presenting a highly infiltrative behaviour usually in single cells (Fig 11). These cells lacked cohesion and appeared individually dispersed through a fibrous connective tissue, sometimes in single linear cords (n=2). This particular histopathological description overlaps the characteristics assigned to human pleomorphic lobular carcinoma, a variant of classical lobular carcinoma. As for sarcomas (n=3), we identified an osteosarcoma and 2 fibrosarcomas. Osteosarcoma was characterized by the presence of polyhedral and pleomorphic neoplastic cells, accompanied by osteoid formation. Fibrosarcomas were composed of highly infiltrative spindle cell proliferations, arranged in interlacing fascicles, associated with marked cellular pleomorphism and the presence of frequent mitotic figures.

For statistical purposes, we grouped malignant tumours in three main groups, considering cell differentiation: simple carcinomas (carcinomas composed of one cell type, which included solid carcinomas, tubulopapillary “simple” carcinomas, anaplastic carcinomas and spindle cell carcinomas), complex carcinomas (carcinomas composed of epithelial and myoepithelial cell proliferation, which included the above described complex carcinomas, in addition to carcinoma in benign tumour and tubulopapillary “complex” carcinoma types), and carcinosarcomas (tumours composed of a carcinoma and a sarcoma component). Sarcoma tumour type was excluded due to the small number of cases.

The presence of intra-tumoural necrosis, usually observed as large necrotic areas (Fig. 12), was observed in 117 (75%) tumour cases, being highly associated with malignancy (105/110 cases). Concerning mode of growth, seventy nine (50.6%) cases showed an infiltrative tumour growth, always found to be associated with a malignant diagnosis.

Table 2. Frequencies observed in the present series for histopathological parameters.

Histopathological parameter	Frequencies n (%)
Histological type (n=156)	
Benign tumours	
Benign mixed tumours	22 (14.1%)
Complex adenoma	13 (8.3%)
Basaloid adenoma	11 (7.1%)
Malignant tumours	
Complex carcinoma	31 (19.9%)
Solid carcinoma	27 (17.3%)
Tubulopapillary carcinoma	19 (12.2%)
Carcinosarcoma	15 (9.6%)
Carcinoma in benign tumour	7 (4.5%)
Spindle cell carcinoma	5 (3.2%)
Anaplastic carcinoma	3 (1.9%)
Sarcoma	3 (1.9%)
Necrosis (n=156)	
Absent	39 (25%)
Present	117 (75%)
Mode of growth (n=156)	
Expansive	77 (49.4%)
Infiltrative	79 (50.6%)
Inflammatory cellular infiltrates (n=156)	
Slight/Absent	58 (37.2%)
Moderate	58 (37.2%)
Marked	40 (25.6%)
Histological grade (n=107)	
Grade I	18 (16.8%)
Grade II	34 (31.8%)
Grade III	55 (51.4%)
Stromal Invasion (n=156)	
Absent	73 (46.8%)
Present	83 (53.2%)
Lymphovascular Invasion (n=156)	
Absent	91 (58.3%)
Present	65 (41.7%)
Lymph node metastasis (n=68)	
Absent	36 (52.9%)
Present	32 (47.1%)

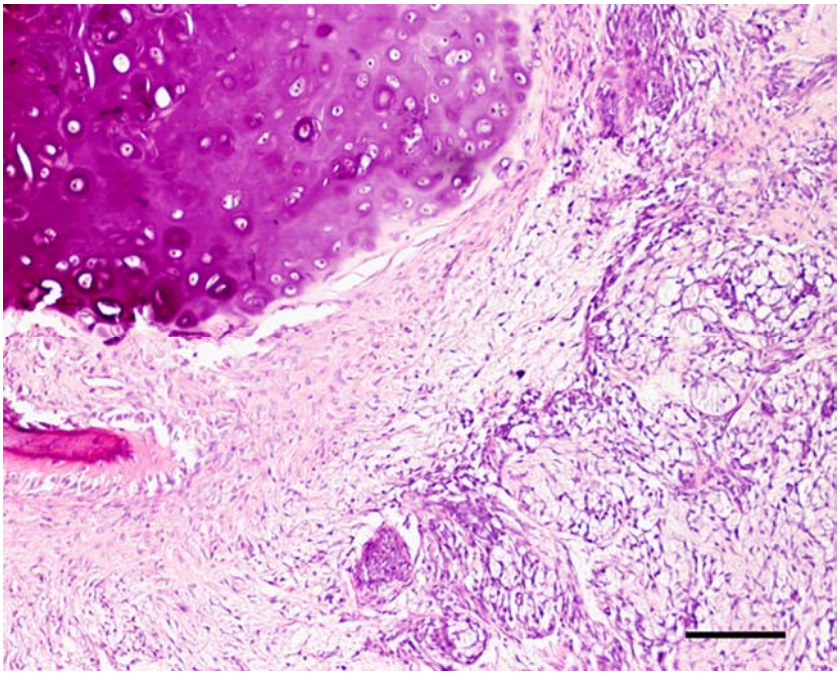

Fig. 2. Mammary gland; dog. Benign mixed tumour characterized by the proliferation of both epithelial and mesenchymal elements (cartilage and bone). HE. Bar=60µm.

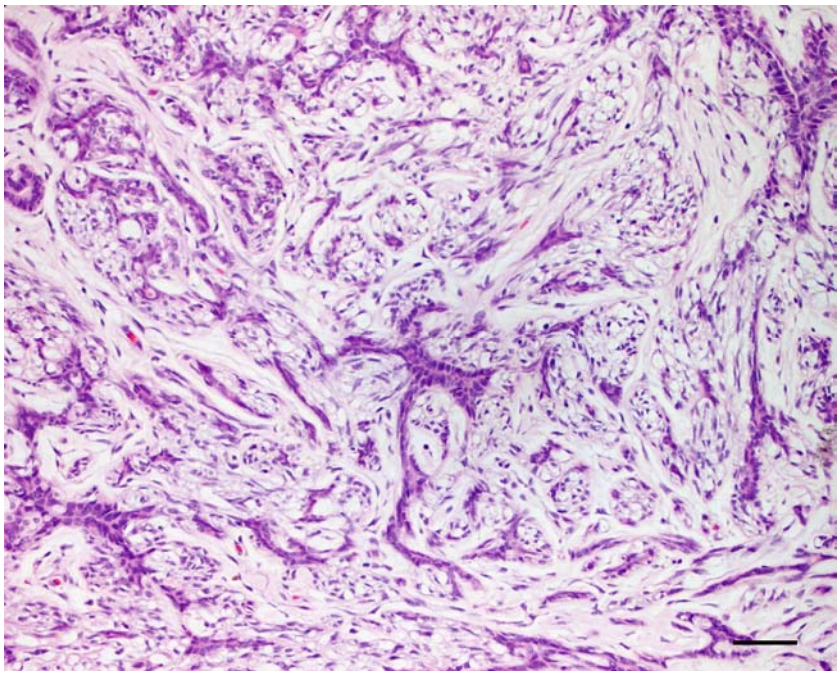

Fig. 3. Mammary gland; dog. Complex adenoma composed by the proliferation of both luminal epithelial and myoepithelial well differentiated cells. HE. Bar=60µm.

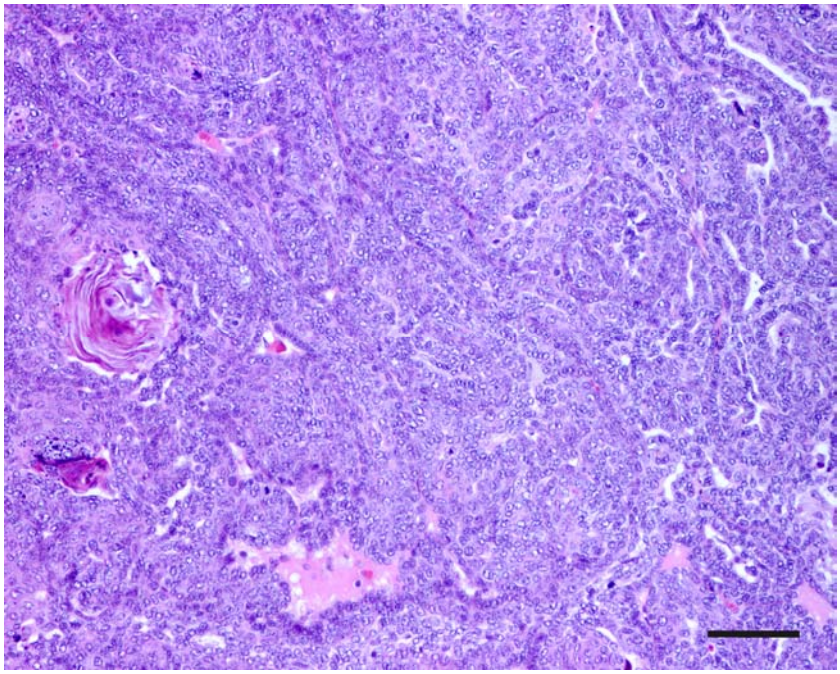

Fig. 4. Mammary gland; dog. Basaloid adenoma characterized by the proliferation of uniform cords or clusters of monomorphic basaloid epithelial cells, showing occasional squamous differentiation. HE. Bar=60µm.

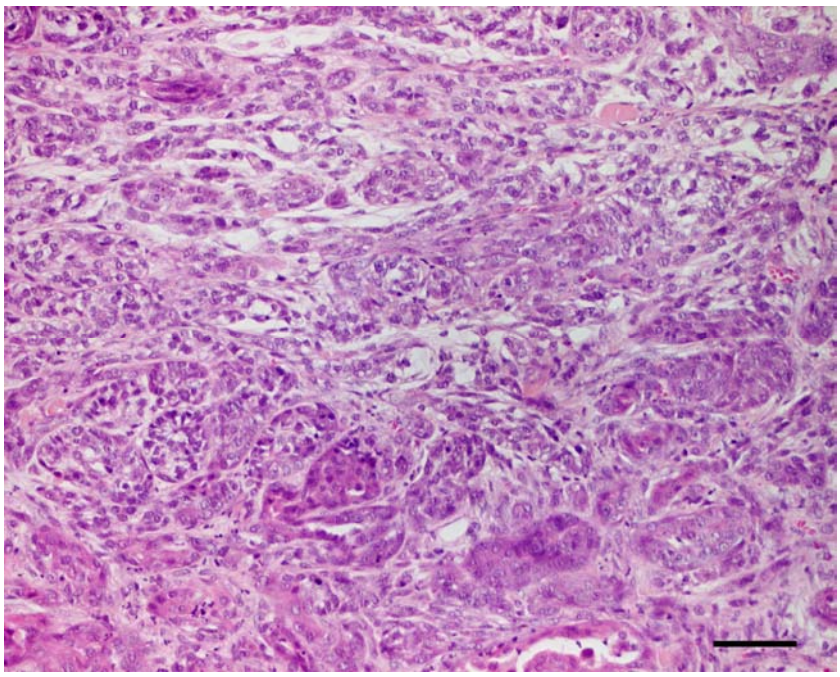

Fig. 5. Mammary gland; dog. Complex carcinoma characterized by the proliferation of both luminal epithelial and myoepithelial cells, exhibiting moderate cellular atypia HE. Bar=60µm.

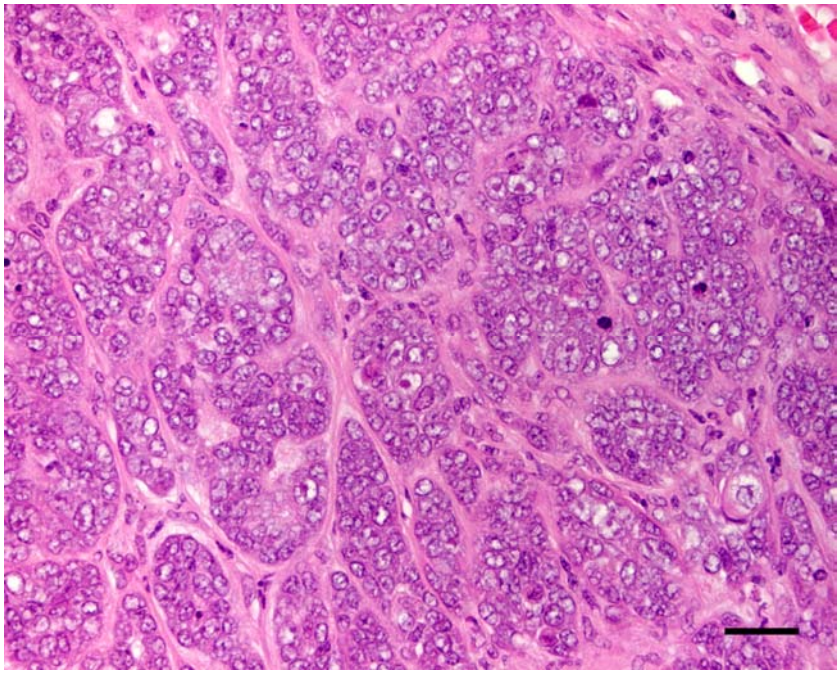

Fig. 6. Mammary gland; dog. Solid carcinoma characterized by the proliferation of epithelial cells arranged in solid nests, associated with marked cellular pleomorfism and high mitotic index. HE. Bar=30µm.

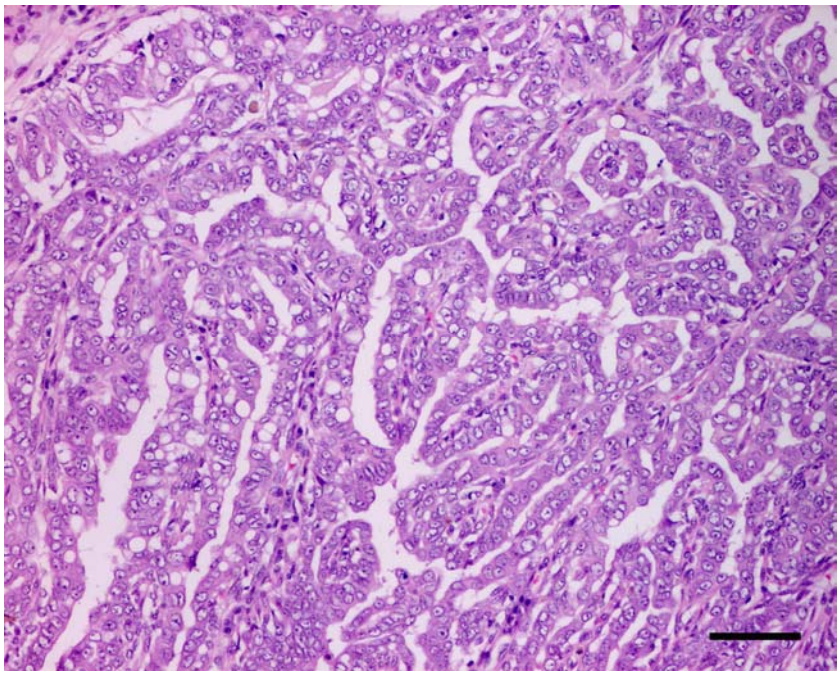

Fig. 7. Mammary gland; dog. Tubulopapillary carcinoma characterized by the proliferation of luminal epithelial cells showing a tubular and/or papillary arrangement. HE. Bar=60µm.

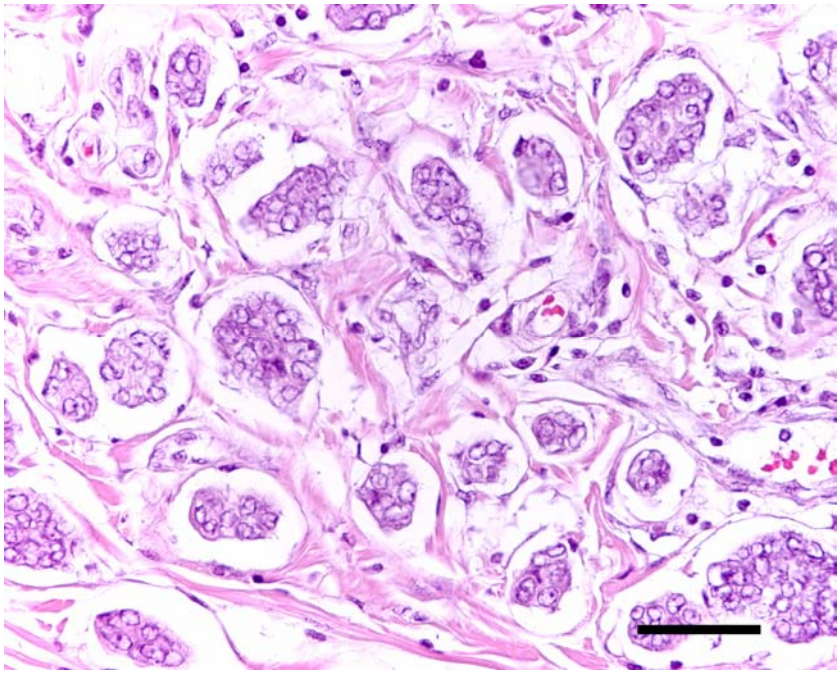

Fig. 8. Mammary gland; dog. Micropapillary carcinoma composed of small papillary cell clusters surrounded by empty lacunar spaces. HE. Bar=40µm.

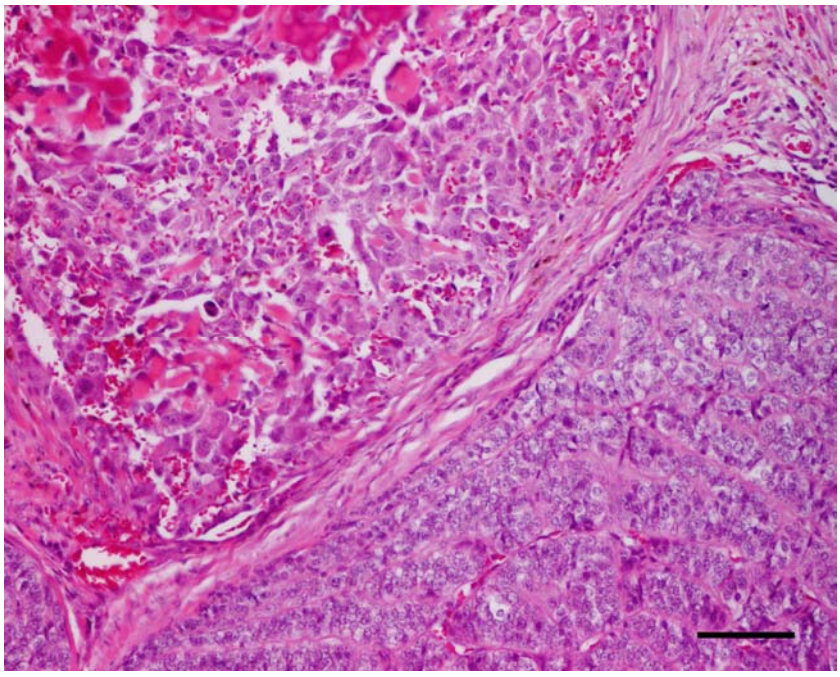

Fig. 9. Mammary gland; dog. Carcinosarcoma showing a sarcoma and a carcinoma component. HE. Bar=60µm.

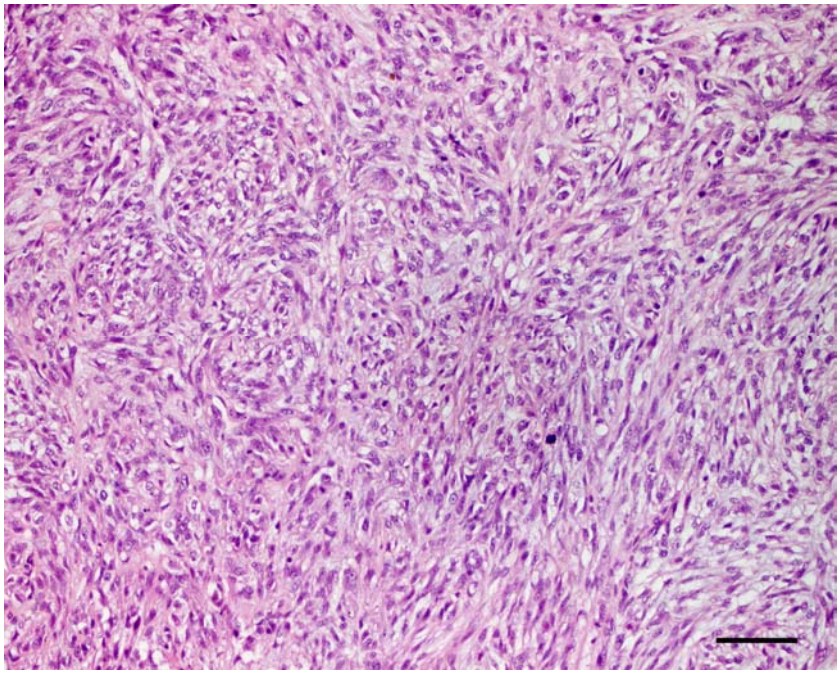

Fig. 10. Mammary gland; dog. Spindle cell carcinoma composed by spindle neoplastic cells. HE. Bar=60µm.

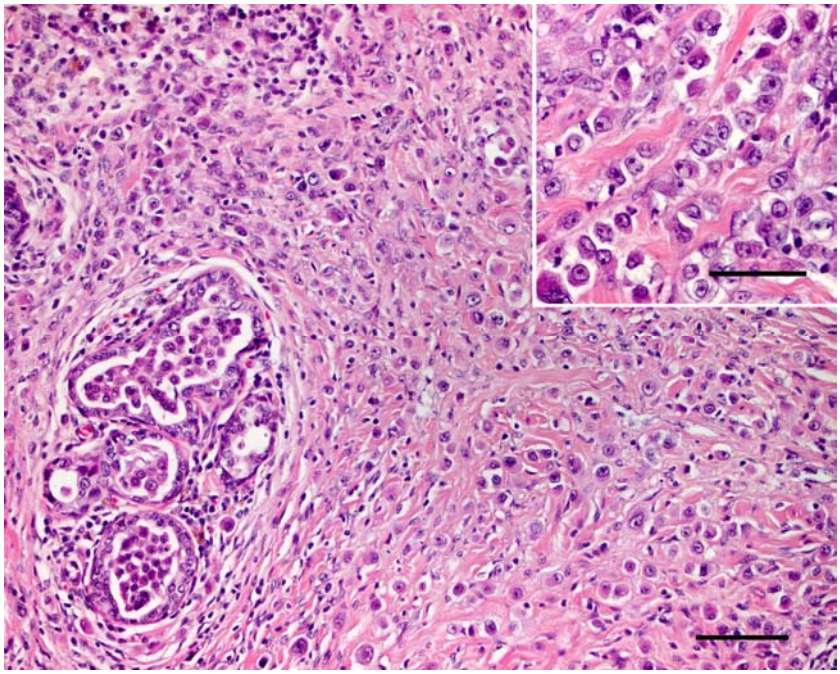

Fig. 11. Mammary gland; dog. Anaplastic carcinoma characterized by the proliferation of infiltrative non-cohesive epithelial cells. HE. Bar=60µm. Inset bar=40 µm.

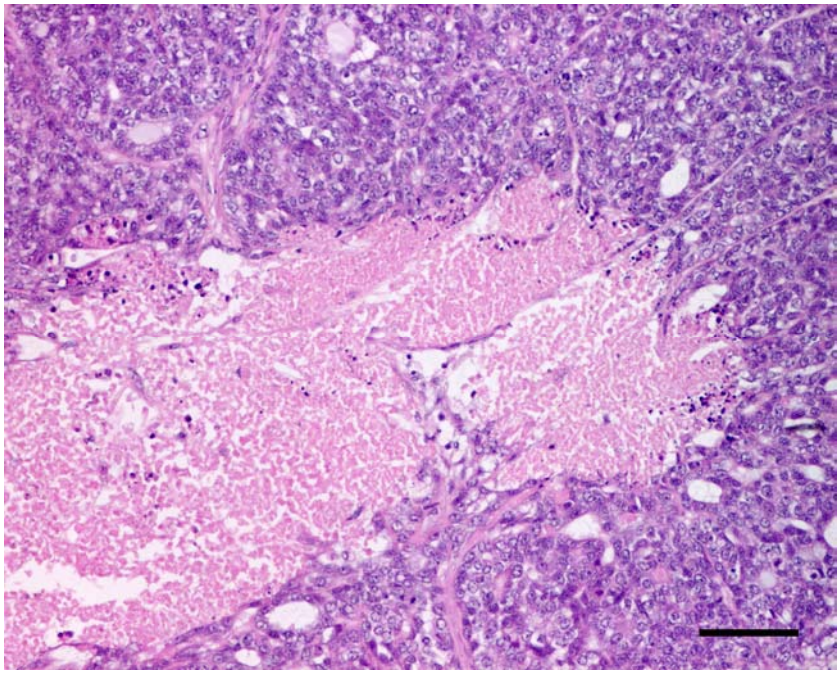

Fig. 12. Mammary gland; dog. Solid carcinoma showing the presence of intra-tumoural necrosis. HE. Bar=60µm.

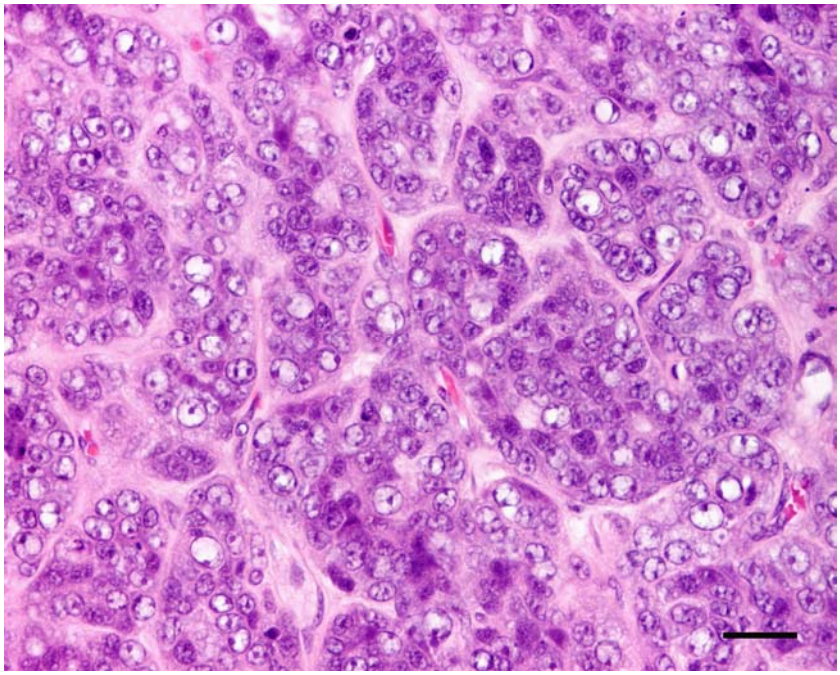

Fig. 13. Mammary gland; dog. Grade III mammary carcinoma, showing marked nuclear pleomorphism. HE. Bar=30µm.

As for inflammatory cellular infiltrates, most benign tumours presented a reduced or absent inflammatory cell infiltration (n=26, 56.5%), whereas malignant ones were usually associated with a moderate inflammatory response (n=43, 39%). When observed, inflammatory infiltrate was mainly characterized by the presence of lymphoid cells (mature lymphocytes and plasma cells).

According to the Nottingham method for histological grading of human breast carcinomas, canine mammary carcinomas (n=107) were classified as grade I (n=18, 16.8%), grade II (n=34, 31.8%) and grade III (n=55, 51.4%) (Fig. 13).

Eighty-three (53.2%) tumours showed stromal invasion, and 65 (41.7%) cases exhibited lymphovascular invasion, all diagnosed as malignant tumours. All carcinosarcoma, spindle cell carcinoma and anaplastic carcinoma cases showed stromal invasion. Anaplastic (n=3, 100%) and solid carcinoma (n=24, 88.9%) types showed the higher levels of vascular invasion.

In our series, we obtained lymph nodes in 68 tumour cases (Fig. 14). From these, 32 (47.1%) have revealed the presence of epithelial cancer cells by histological evaluation. Solid carcinoma (n=15, 78.9%) and anaplastic carcinoma (n=3, 100%) were the most frequent tumour types associated with positive lymph node metastasis.

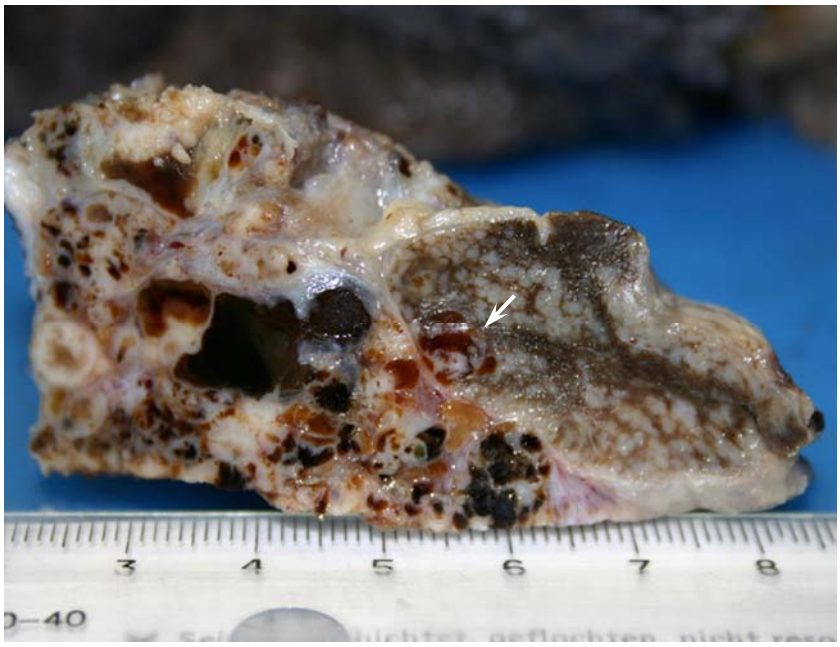

Fig. 14. Female dog, mastectomy specimen. Regional lymph node metastasis (arrow).

Statistical analysis

Tables 3 and 4 elucidate the differences observed between benign and malignant tumours, with regard to the evaluated variables. Proliferation mitotic and Ki-67 labelling indices are presented in Table 4. Ki-67 evaluation was possible in 137 cases (Fig 15) (19 cases showed no immunostaining and were excluded of the analysis). Based on the median values of these indices, a significant difference was observed between benign and malignant tumours.

Table 3. Association between clinical parameters and histological diagnosis observed in the present series

Clinical parameter	Benign n (%)	Malignant n (%)
Age (n=146)		
≤9 years	26 (35.1%)	48 (64.9%)
>9 years	16 (22.2%)	56 (77.8%)
	P=0.1	
Breed (n=145)		
Mixed breed	12 (25%)	36 (75%)
Poodle	7 (28%)	18 (72%)
Cocker spaniel	5 (29.4%)	12 (70.6%)
Others	17 (30.9%)	38 (69.1%)
	P=0.92	
Ovariohysterectomy (n=98)		
No	15 (21.4%)	55 (78.6%)
Yes, prior to tumour development	2 (15.4%)	11 (84.6%)
Yes, performed with mastectomy	2 (13.3%)	13 (86.7%)
	P=0.78	
Contraception (n=98)		
No	13 (18.1%)	59 (81.9%)
Yes	0 (0%)	8 (100%)
	P=0.34	
Tumour size (n=149)		
<3 cm	42 (52.5%)	38 (47.5%)
3-5 cm	4 (9.8%)	37 (90.2%)
>5 cm	0 (0%)	28 (100%)
	P<0.0001	
Tumour location (n=89)		
Cranial glands (thoracic)	1 (12.5%)	7 (87.5%)
Medial gland (cranial abdominal)	2 (14.3%)	12 (85.7%)
Caudal glands (caudal abdominal and inguinal)	11 (22%)	39 (78%)
Multiple	1 (5.9%)	16 (94.1%)
	P=0.47	
Skin ulceration (n=154)		
No	46 (34.6%)	87 (65.4%)
Yes	0 (0%)	22 (100%)
	P<0.0001	

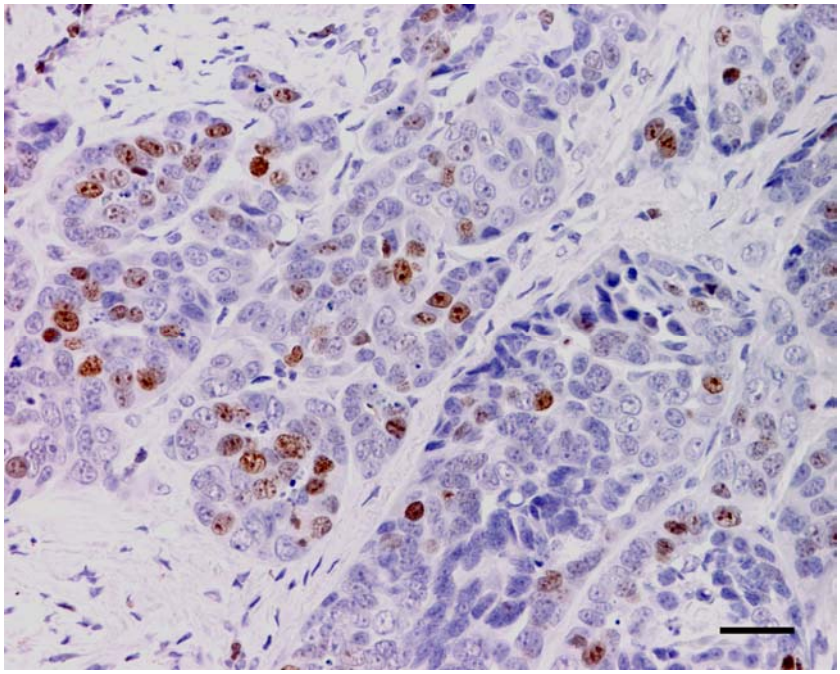

Fig. 15. Mammary gland; dog. Solid carcinoma showing nuclear Ki-67 positive immunostaining. Bar=30μm.

Malignant tumours were found to be associated with increased tumour size (P<0.0001), presence of skin ulceration (P<0.0001) and necrosis (P<0.0001), infiltrative tumour growth (P<0.0001) and a marked inflammatory cell response (P=0.006). Stromal/vascular invasion and lymph node metastases were restricted to malignant lesions. As for proliferation, these tumours also showed high mitotic (P<0.0001) and Ki-67 (P<0.0001) labelling indices.

Considering the malignant tumour group, several differences were observed across distinct histological types (Table 5). Simple carcinomas and carcinosarcomas showed a larger tumour size, whereas complex carcinomas were significantly smaller (P=0.044). In addition, the latter group was significantly associated with an expansive growth (P<0.0001), low differentiation grade (P<0.0001), reduced stromal (P<0.0001) and vascular invasion (P<0.0001). Simple carcinomas and carcinosarcomas were significantly associated with the presence of node metastases (P<0.0001). As for proliferation, complex carcinomas presented the lowest labelling indices, whereas the other histological types were significantly associated with increased proliferative mitotic and Ki-67 indices.

Table 4. Associations between histopathological parameters and histological diagnosis observed in the present series.

Histopathological parameter	Benign n (%)	Malignant n (%)
Necrosis (n=156)		
Absent	34 (87.2%)	5 (12.8%)
Present	12 (10.3%)	105 (89.7%)
	P<0.0001	
Mode of growth (n=156)		
Expansive	46 (59.7%)	31 (40.3%)
Infiltrative	0 (0%)	79 (100%)
	P<0.0001	
Inflammatory cellular infiltrates (n=156)		
Slight/Absent	26 (44.8%)	32 (55.2%)
Moderate	15 (25.9%)	43 (74.1%)
Abundant	5 (12.5%)	35 (87.5%)
	P=0.002	
Stromal Invasion (n=156)		
Absent	46 (63%)	27 (37%)
Present	0 (0%)	83 (100%)
	P<0.0001	
Lymphovascular Invasion (n=156)		
Absent	46 (50.5%)	45 (49.5%)
Present	0 (0%)	65 (100%)
	P<0.0001	
Lymph node metastasis (n=68)		
Absent	8 (22.2%)	28 (77.8%)
Present	0 (0%)	32 (100%)
	P=0.006	
Mitotic Index		
Median (Minimum-Maximum)	0.2 (0-1.47)	0.7 (0-2.99)
	P<0.0001	
Ki-67 Index		
Median (Minimum-Maximum)	11.86 (6.81-26.93)	23.5 (5.39-56.36)
	P<0.0001	

Kaplan-Meier univariate analysis

Follow-up data concerning OS was available in 69 bitches with malignant tumours. During the follow-up period, according to the referring surgeons, 37 animals died or were euthanized due to metastatic disease and/or local recurrence (25 with distant metastases, 7 with local recurrence and 5 with both recurrence and distant metastases).

Table 5. Significant differences found between distinct malignant groups in the present series.

Clinicopathological parameter	Simple carcinoma	Complex carcinoma	Carcinosarcoma
Tumour size (n=100)			
<3 cm	13 (35.1%)	22 (59.5%)	2 (5.4%)
3-5 cm	18 (51.4%)	13 (37.1%)	4 (11.4%)
>5 cm	15 (53.6%)	7 (25%)	6 (21.4%)
	P=0.044		
Mode of growth (n=110)			
Expansive	4 (12.9%)	27 (87.1%)	0 (0%)
Infiltrative	43 (56.6%)	18 (23.7%)	15 (19.7%)
	P<0.0001		
Histological grade (n=107)			
Grade I	1 (5.6%)	16 (88.9%)	1 (5.6%)
Grade II	9 (26.5%)	23 (67.6%)	2 (5.9%)
Grade III	37 (27.3%)	6 (10.9%)	12 (21.8%)
	P<0.0001		
Stromal Invasion (n=110)			
Absent	4 (14.8%)	23 (85.2%)	0 (0%)
Present	43 (53.8%)	22 (27.5%)	15 (18.8%)
	P<0.0001		
Lymphovascular Invasion (n=110)			
Absent	6 (13.3%)	35 (77.8%)	4 (8.9%)
Present	41 (66.1%)	10 (16.1%)	11 (17.7%)
	P<0.0001		
Lymph node metastasis (n=57)			
Absent	7 (26.9%)	18 (69.2%)	1 (3.8%)
Present	25 (80.6%)	1 (3.2%)	5 (16.1%)
	P<0.0001		
Mitotic Index			
Median (Min-Max)	0.96 (0.1-2.99)	0.46 (0.0-1.6)	0.8 (0.1-1.90)
	P<0.0001		
Ki-67 Index			
Median (Min-Max)	27.5 (12.10-	17.84 (5.39-	27.2 (10.2-40.9)
	P<0.0001		

Distant metastases were predominantly found in the lung (n=28) and liver (n=8) (Fig. 16 and 17). At 12 months after mastectomy, 33 (47.83%) animals have died whereas 36 of the 69 (52.17%) dogs enrolled in the follow-up study were alive; the median overall survival was 15 months. Tables 6 and 7 present those factors significantly associated with OS and DFS, respectively.

Statistically significant associations were achieved between OS and size (P=0.0042), skin ulceration (P=0.0322), tumour growth (P<0.0001), stromal and lymphovascular invasion (P<0.0001 and P<0.0001, respectively), lymph node status (P<0.0001),

mitosis (*P*=0.0143) and Ki-67 (*P*=0.0001). Histological type and grade were also significantly related to OS. Dogs with simple or carcinosarcoma histotype showed poorer OS times (*P*<0.0001) than did the ones affected by complex carcinomas. Survival was significantly worse in grade III cases (*P*<0.0001), compared to grade I/II.

Table 6. Factors significantly associated to overall survival in malignant tumours.

Variable	n	Overall survival		*P*
		Mean survival (months)	Average 1-year survival rate (n[%])	
Tumour size				
<3 cm	27	48.63	19 (70.37)	0.0042
3-5 cm	17	19.59	9 (52.94)	
>5 cm	19	10.21	5 (26.32)	
Skin ulceration				
No	55	37.41	31 (56.36)	0.032
Yes	13	10.62	5 (38.46)	
Histological type				
Simple carcinoma	24	8.21	6 (25)	<0.0001
Complex carcinoma	36	49.68	28 (77.78)	
Carcinosarcoma	9	7.33	2 (22.22)	
Mode of growth				
Expansive	24	58.14	22 (91.67)	<0.0001
Infiltrative	45	9.08	14 (31.11)	
Histological grade				
Grade I/II	36	47.43	28 (77.78)	<0.0001
Grade III	33	9.38	8 (24.24)	
Stromal Invasion				
Absent	20	57.88	19 (95)	<0.0001
Present	49	13.91	17 (34.69)	
Lymphovascular Invasion				
Absent	32	56.09	29 (90.63)	<0.0001
Present	37	6.57	7 (18.92)	
Lymph node metastasis				
Absent	15	39.74	13 (86.67)	<0.0001
Present	15	6.53	3 (20)	
Mitotic index				
<0.7	35	39.24	25 (71.43)	0.0143
≥0.7	29	17.86	9 (31.03)	
Ki-67 index				
<23.5	35	47.62	26 (74.29)	0.0001
≥23.5	29	13.35	8 (27.59)	

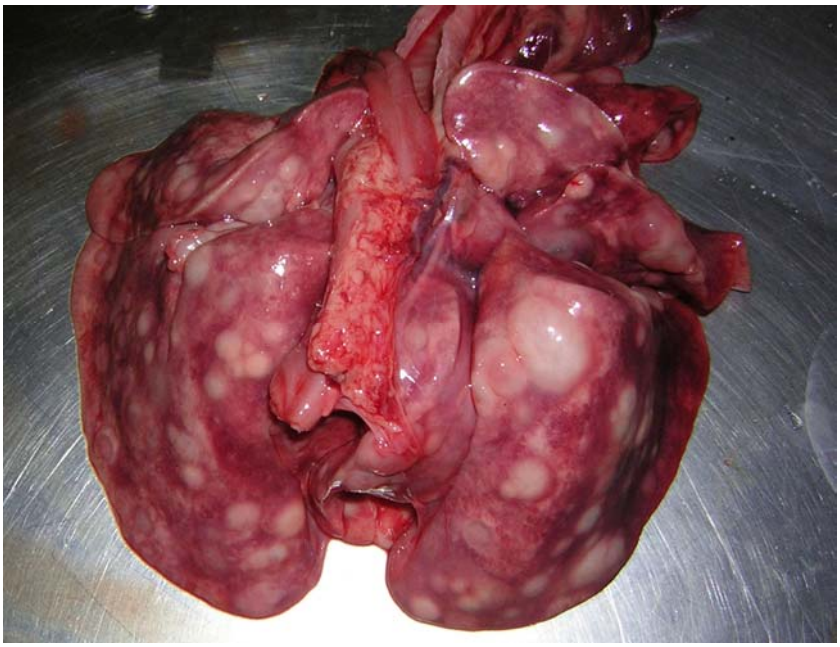

Fig. 16. Female dog. Lung showing multiple metastases of a mammary carcinosarcoma.

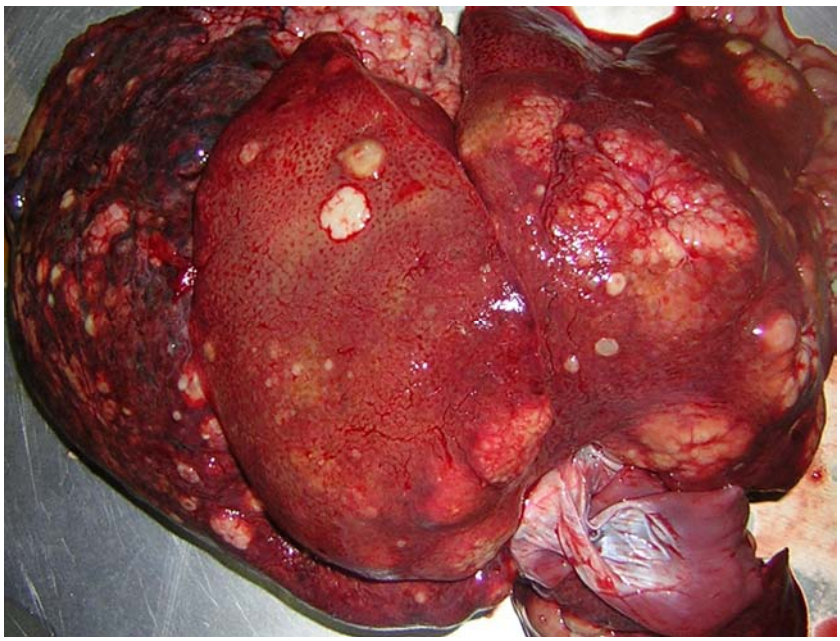

Fig. 17. Female dog. Liver showing multiple metastases of a mammary carcinoma.

Data for DFS was available in 68 cases. One case was excluded from this study because the bitch developed oncologic disease within 10 to 30 days after surgery. At 12 months after mastectomy, 33.8% (23/68) of dogs were free of oncological disease; the median disease-free survival was 9 months. Statistically significant differences were achieved between DFS and tumour size (P=0.04), histological type (P=0.0037), tumour growth (P<0.0001), grade (P=0.002), stromal and lymphovascular invasion (P=0.0018 and P<0.0001, respectively), lymph node invasion on clinical presentation (P=0.0017), mitosis (P=0.0134) and Ki-67 labelling index (P=0.0005).

Table 7. Factors significantly associated to disease-free survival in malignant tumours.

Variable	n	Disease-free survival		*P*
		Mean survival (months)	Average 1-year survival rate (n[%])	
Tumour size				
<3 cm	27	14.56	13 (48.15)	0.04
3-5 cm	17	17.82	8 (47.06)	
>5 cm	18	6.22	2 (11.11)	
Histological type				
Simple carcinoma	23	7.22	6 (26.09)	0.0037
Complex carcinoma	36	17.88	17 (47.22)	
Carcinosarcoma	9	5.78	0 (0)	
Mode of growth				
Expansive	24	22.33	15 (62.5)	<0.0001
Infiltrative	44	6.86	8 (18.18)	
Histological grade				
Grade I/II	36	17.31	16 (44.44)	0.002
Grade III	32	7.97	7 (21.88)	
Stromal Invasion				
Absent	20	18.46	12 (60)	0.0018
Present	48	10.05	11 (22.92)	
Lymphovascular Invasion				
Absent	32	22.05	19 (59.38)	<0.0001
Present	36	4.97	4 (11.11)	
Lymph node metastasis				
Absent	15	17.93	9 (60)	0.0017
Present	14	4.93	2 (14.29)	
Mitotic index				
<0.7	35	17.29	15 (42.86)	0.0134
≥0.7	28	8.56	7 (25)	
Ki-67 index				
<23.5	35	18.35	17 (48.57)	0.0005
≥23.5	28	7.17	5 (17.86)	

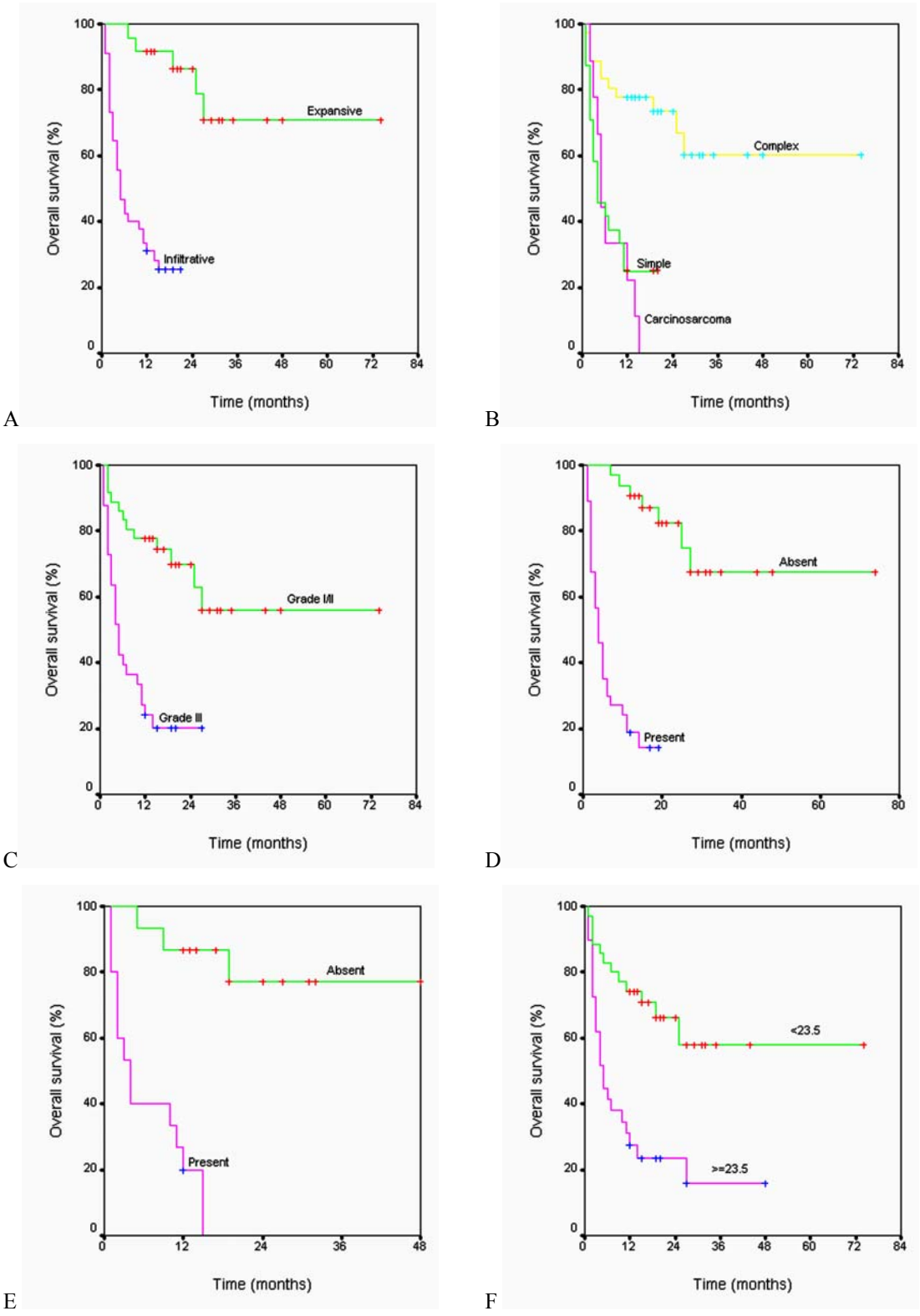

Fig. 18. Overall Kaplan-Meier survival curves for dogs with malignant mammary tumours. A - Tumour growth (expansive vs. infiltrative), P<0.0001; B - Histological type (complex vs. simple vs. carcinosarcoma), P<0.0001; C- Histological grade (Grade I/II vs. Grade III), P<0.0001; D- Lymphovascular invasion (Absent vs. present), P<0.0001; E- Lymph node metastases (Absent vs. present), P<0.0001; F- Ki-67 labelling index (high vs. low index), P=0.0001.

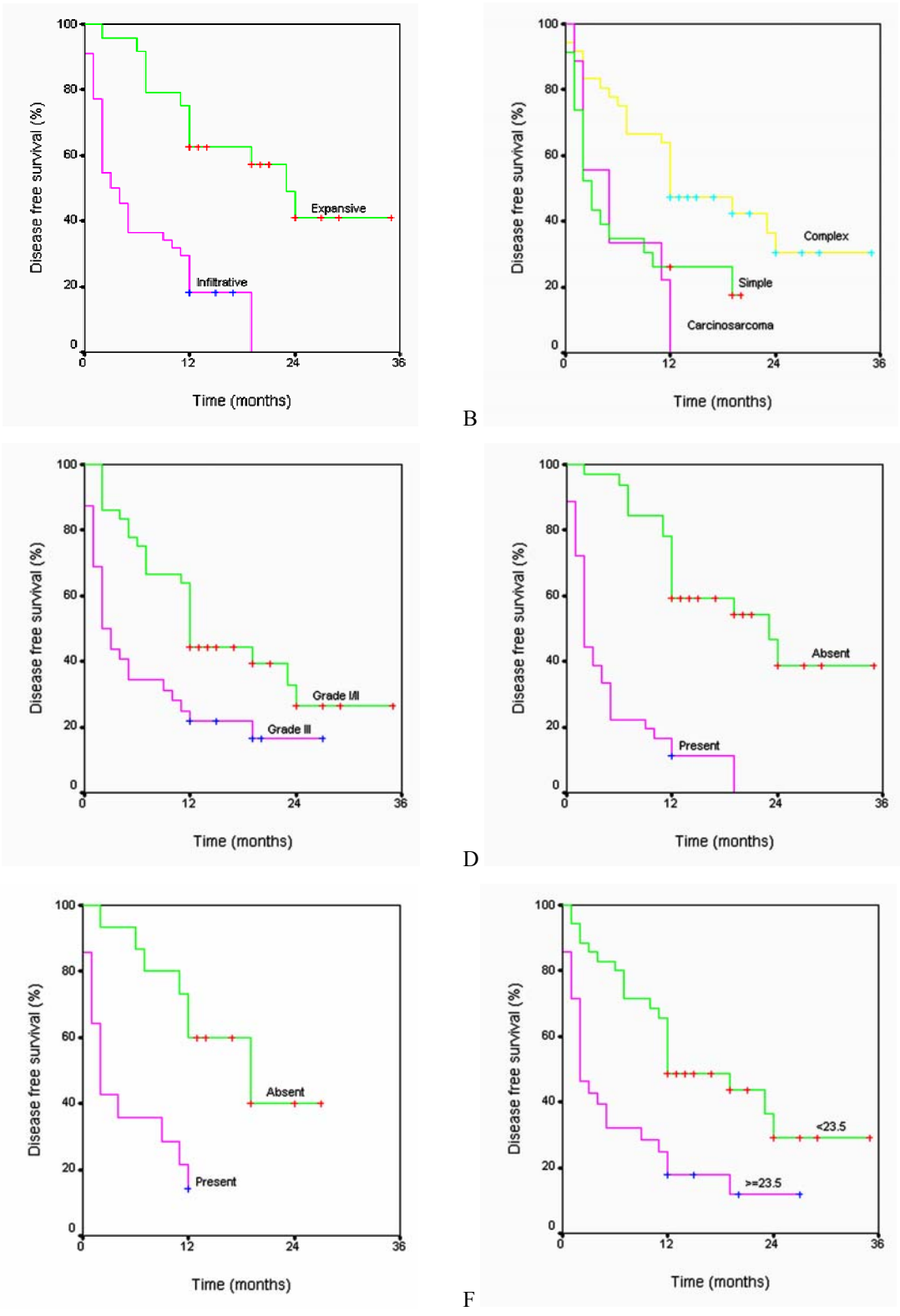

Fig. 19. Disease-free Kaplan-Meier survival curves for dogs with malignant mammary tumours. A - Tumour growth (expansive vs. infiltrative), P<0.0001; B - Histological type (complex vs. simple vs. carcinosarcoma), P=0.0037; C- Histological grade (Grade I/II vs. Grade III), P=0.002; D- Lymphovascular invasion (Absent vs. present), P<0.0001; E- Lymph node metastases (Absent vs. present), P=0.0017; F- Ki-67 labelling index (high vs. low index), P=0.0005.

Multivariate analysis, proportional hazard model

Multivariate analysis including factors significantly associated with survival by the log-rank test was performed using forward Cox regression method. Multivariate analysis disclosed lymph node status as significantly associated with overall and disease-free survival (Table 8). The inclusion of this variable reduced the number of available observations considerably because of missing data, and therefore models without this variable were also estimated, confirming lymphovascular invasion as an independent prognostic factor for both overall (HR: 19.33; 95% CI: 5.65-66.15; $P<0.0001$) and disease-free survival (HR: 6.75; 95% CI: 3.19-14.25; $P<0.0001$).

Table 8. Significant prognostic factors in multivariate analysis for dogs with malignant tumours.

Dependent variable	Independent variables	Hazard ratio (HR)	95% CI	*P*
Overall survival	Lymph node metastases			
	Absent	Referent		
	Present	10.924	2.38-50.12	0.002
Disease-free survival	Lymph node metastases			
	Absent	Referent		
	Present	4.494	1.50-13.46	0.007

Discussion

In this study we have characterized a series of benign and malignant tumours at the clinical and pathological level and we performed an evaluation of proliferation labelling indices. These variables were compared across benign and malignant tumour types and a survival analysis was conducted in order to improve understanding of prognostic factors in canine mammary tumours. Determining the prognosis of a canine patient with a malignant mammary tumour is very important for the clinician, but it is often difficult because the biologic behaviour of these tumours varies widely. The major challenge is to find those prognostic variables that allow the prediction of disease behaviour in the individual case (Sarli *et al*., 2002).

A diagnosis of malignancy was significantly associated with clinical and pathological aggressive features. In our series, increased tumour size and ulceration were significantly associated with tumour malignancy, with all tumours larger than 5cm and presenting skin ulceration classified as malignant. Univariate analysis of survival

showed that these clinical variables were of prognostic value on OS, confirming previous studies (Bostock, 1975; Misdorp and Hart, 1976; Shofer *et al*., 1989; Yamagami *et al*., 1996b; Matos, 2007). Tumour size was also significantly associated with DFS, which was also described by others (Peña *et al*., 1998; Martin de las Mulas *et al*., 2005). In our study, ulceration was not found of prognostic value on disease-free survival, which is contrast with previous studies (Hellmén *et al*., 1993; Peña *et al*., 1998; Queiroga and Lopes, 2002).

We have found no differences between benign and malignant tumours with regard to animal age at the time of surgical procedure. Also, no statistical significant association was observed with clinical outcome. The available literature shows similar mean age values to our study (around 9-10 years), but opposing results exists concerning its prognostic information; although several investigations described animal age as a prognostic factor, with old animals associated with more aggressive tumours (Schneider *et al*., 1969; Hellmén *et al*., 1993; Peña *et al*., 1998; Nieto *et al*., 2000), others have not confirmed these findings (Philibert *et al*., 2003; Martin de las Mulas *et al*., 2005; Matos, 2007). These contradictory results might be related with differences in statistical approaches (some studies have considered age as a continuous and others as a categorical variable) or with animal age variations between studies.

No significant associations were found between other clinical variables and tumour diagnosis or outcome. However, an association was recently described between small animal breeds and a lower rate of tumour malignancy (Itoh *et al*., 2005), when comparing small vs. other breeds. Although ovariohysterectomy has been decribed as having a protective effect on the development of mammary tumours and some studies have reported its association with increased survival times (Sorenmo *et al*., 2000; Chang *et al*., 2005), the prognostic value of this procedure is still under debate. Similarly to the present findings, other researchers found no association between ovariohysterectomy before or at the time of mastectomy and patient prognosis (Morris *et al*., 1998; Philibert *et al*., 2003). In addition, no association between the administration of contraceptives and malignancy or outcome was observed, as previously reported by other studies (Hellmén *et al*., 1993; Peña *et al*., 1998; Nieto *et al*., 2000; Martin de las Mulas *et al*., 2005).

It is obvious from the available literature the controversy around histological classification of canine mammary tumours. Several classification systems have been

proposed in the last decades (Fowler *et al*., 1974; Bostock, 1975; Moulton, 1990) and the published studies parallel these distinct criteria. Diverse histological categorizations have been used, rendering a comparison between studies difficult. With the recent proposed WHO classification, a prognostic element has been introduced, separating complex and simple carcinomas, the latter characterized by higher malignancy (Misdorp *et al*., 1999). Considering this classification system and given that we had a small number of samples in some histological types, we have grouped carcinomas into these two main groups of carcinomas and we have considered a third group, composed only by those tumours fulfilling malignant criteria in both epithelial and mesenchymal tumour components (carcinosarcoma group). When we considered these 3 major groups of malignant tumours, we found that complex carcinomas were associated with less aggressive pathological features such as small size, expansive tumour growth, low to moderate histological grade, lack of stromal and vascular invasion and reduced number of lymph node metastases. Accordingly, this tumour group was significantly associated with the lowest proliferation indices. These characteristics were reflected on survival analysis, which revealed better overall and disease-free survival times for this type of neoplasms. Despite some differences in tumour categorization, our results are in conformity with other studies, who also found complex carcinomas as the ones showing a more favourable clinical behaviour (Misdorp and Hart, 1976; Yamagami *et al*., 1996b; Matos, 2007). However, recent studies did not found a statistical significant association between histological type and disease-free survival (Martin de las Mulas *et al*., 2005), probably because of differences in tumour categories.

Complex lesions of the present series were characterized by a myoepithelial component, usually readily identified on HE stained sections, admixed with the proliferation of luminal epithelial cells. The participation of myoepithelial cells has been associated with the better prognosis assigned to complex carcinomas (Yamagami *et al*., 1996b), giving that these cells have been described as having a tumour/invasive suppressor function in several human studies (Sternlicht *et al*., 1997; Barsky, 2003; Jones *et al*., 2003; Adriance *et al*., 2005). However, although these biphasic tumours (myoepithelial and epithelial differentiation) are associated with low malignancy in canine (Yamagami *et al*., 1996b), feline (Seixas *et al*., 2008) and human (Foschini and Eusebi, 1998) species, pure malignant myoepithelioma show a distinct clinical behaviour, related with poorer outcome (Foschini and Eusebi, 1998). Accordingly, the spindle cell carcinoma of

our series submitted to follow up (n=3) showed an aggressive behaviour, with overall survival times ranging from 4 to 10 months. Although several recent studies have drawn attention to mammary myoepithelial cells, this cell type has been largely neglected and our understanding of the functions of this second major mammary cell population in mammary gland tumorigenesis remains very limited (Faraldo *et al*., 2005).

In contrast to complex carcinomas, simple carcinoma and carcinosarcoma types showed very aggressive features, as described by previous studies (Misdorp and Hart, 1976; Misdorp *et al*., 1999; Sorenmo, 2003; Matos, 2007). Simple carcinoma group comprised a variety of histological types, but a small number of cases per type had available follow-up in order to perform a consistent statistical analysis. Future studies are needed in order to compare their biological behaviour. One histological type that deserves further attention is the micropapillary invasive carcinoma, which have been associated with a very aggressive behaviour in canine (Cassali *et al*., 2002b; Gama *et al*., 2008) and feline (Seixas *et al*., 2007) species, as well as in humans (Siriaunkgul and Tavassoli, 1993; Kuroda *et al*., 2004; Kim *et al*., 2005; Nassar, 2004; Putti *et al*., 2005). In the present series, 3 cases were described, all associated with rapid progression of the oncologic disease. Only one case was included in the follow-up study, since the other 2 dogs died immediately after surgery. Another rare histological type found in our tumour series (n=2) was the pleomorphic lobular carcinoma, a recognized subtype of invasive lobular carcinoma described in the human species (Eusebi *et al*., 1992; Radhi, 2000). One case of canine pleomorphic lobular carcinoma has been reported previously by Cassali *et al*. (2002), which described similar cytomorphologic features to the ones found in our cases (Cassali *et al*., 2002a).

With regard to histological grade, grade III tumours were found significantly associated with simple carcinoma and carcinosarcoma types, shorter overall and disease-free survival rates, when compared to grade I and II tumours. Our results are in accordance to previous reports on canine (Karayannopoulou *et al*., 2005; Martin de las Mulas *et al*., 2005; Matos, 2007) and human (Elston and Ellis, 1991) tissues.

In our study, the presence of tumour necrosis was associated with a malignant phenotype but it was not associated with decreased survival intervals. This result is similar to a recent study (Matos, 2007) but contradicts another, in which necrosis was associated with a shorter disease-free survival (Martin de las Mulas *et al*., 2005).

An infiltrative growth pattern and stromal invasion were restricted to the malignant tumour group, being significantly associated with simple and carcinosarcoma histological types, as well as with poorer survival times. This result is in accordance to some studies (Misdorp and Hart, 1976; Sarli *et al*., 2002), but contradicts others who failed to find differences between tumour growth pattern or stromal invasion and prognosis (Itoh *et al*., 2005; Martin de las Mulas *et al*., 2005).

Lymphoid cellular infiltration was associated with malignancy, but no other associations were found. It was suggested that lymphoid cellular reactivity could indicate an anti-tumour immune response and it was associated with a better prognosis (Gilbertson *et al*., 1983), but several studies have also failed in finding such an association (Martin de las Mulas *et al*., 2005; Matos, 2007).

In univariate analysis, lymphovascular invasion was significantly associated with simple and carcinosarcoma tumour types, and strongly associated with local recurrence/distant metastases and decreased overall survival rates. Dogs affected by tumours showing no lymphovascular invasion had a significant survival advantage and a reduced risk of relapse. Our findings confirm previous reports both on canine (Gilbertson *et al*., 1983; Yamagami *et al*., 1996b; Martin de las Mulas *et al*., 2005) and human breast cancer (Pinder *et al*., 1994; Elston *et al*., 1998). However, other authors failed to find such an association (Misdorp and Hart, 1976).

Our results also showed that tumour growth fraction, as assessed by mitotic and Ki-67 labelling indices, is an important predictor of survival. Ki-67 labelling index values of more than 23.5% were significantly associated with shorter overall and disease-free survival times. This comes in accordance with reported findings in canine (Peña *et al*., 1998; Nieto *et al*., 2000; Sarli *et al*., 2002; de Matos *et al*., 2006) and human (Bouzubar *et al*., 1989; Brown and Gatter, 1990; Veronese and Gambacorta, 1992; Pinder *et al*., 1995) tissues, which described Ki-67 as a valuable prognostic factor.

Lymph node status was of statistically significant prognostic value, confirming several previous canine (Gilbertson *et al*., 1983; Nieto *et al*., 2000; Itoh *et al*., 2005; Martin de las Mulas *et al*., 2005) and human breast cancer studies (Elston *et al*., 1998; Ellis *et al*., 2003). Despite the identification of several parameters with prognostic value on univariate analysis, their prognostic power was not retained in the Cox regression multivariate analysis, which considered lymph node status as the only independent variable. As already confirmed by previous studies, this parameter is obviously of great

importance in predicting clinical outcome of bitches with malignant mammary tumours, but several studies failed on proving its prognostic value in multivariate analysis (Misdorp and Hart, 1976; Hellmén *et al*., 1993; Martin de las Mulas *et al*., 2005). A number of reasons have been listed to explain these results: the small number of lymph nodes evaluated (Martin de las Mulas *et al*., 2005), the inclusion of sarcomas (which probably attenuate the effect of node status, giving that sarcomas are usually associated with haematogeneous spreading, rather than lymphatic) (Hellmén *et al*., 1993) or even the particular characteristics of the lymph node network associated with the canine mammary gland (Misdorp and Hart, 1976). Additional studies are warranted evaluating a larger series in order to confirm our present results.

If lymph node status was not included in the multivariate analysis, lymphovascular invasion replaced it as the most important predictive factor for survival in this group of dogs. Sarli *et al*. (2002) also found lymphovascular invasion as an independent prognosticator, but the study performed by Yamagami and co-workers (1996b) did not revealed this significance, despite describing this variable as a prognostic factor in univariate analysis (Yamagami *et al*., 1996b). The most likely explanation for such discrepancies is related to problems in the distinction of true vessels, especially lymphatics, from artefactual soft tissue spaces due to fixation shrinkage artefact (Elston *et al*., 1998; Martin de las Mulas *et al*., 2005).

Survival time is considered a useful criterion for evaluating prognosis in both man and animals (Misdorp, 1987). Although, in our study, several parameters were considered as useful prognostic factors, the present results should be interpreted with caution given the relatively small number of cases with follow-up. All cases were treated by surgery alone and followed for a minimum period of 12 months. During this time, all occurring deaths (n=33) were tumour-related and no dog was lost to follow-up. Since a number of cases are still being submitted to follow-up (12 dogs with a follow-up period inferior to 24 months), we have presented the survival rates observed one year after surgical treatment. However, it is our objective to extend the post-surgical evaluation in order to confirm the validity of our first results.

Canine mammary tumours with apparent signs of malignancy do not present any diagnostic problems for experienced clinicians. However, because many canine tumours are not at an advanced stage of development when first detected by owners or clinicians, the potential biologic behaviour of these tumours is rather difficult to predict (Hellmén

et al., 1993). Pathologists certainly represent a signicant role in the management of dogs with mammary cancer, by providing reliable prognostic information to clinicians. From our preliminary study, lymph node status was the only independent prognostic factor in canine mammary malignant tumours; unfortunately, a considerable number of mastectomy and lumpectomy specimens received in the histopathology laboratories are not accompanied by regional lymph nodes, fact that was reflected on our and other studies. We believe that it is of major importance to encourage veterinary surgeons to routinely remove lymph nodes whenever they perform a mastectomy, even if no clinical signs of metastases are observed.

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Chapter III

Expression of E-cadherin, P-cadherin and β-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival.

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Expression of E-cadherin, P-cadherin and β-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival

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Abstract

Cadherin–catenin complexes play a critical role in intercellular adhesion, and their altered expression has been implicated in tumour progression. In this study, the expression of E-cadherin, P-cadherin and β-catenin was analysed in 65 canine malignant mammary tumours and correlated with clinicopathological parameters, proliferation and survival. Reduction in E-cadherin expression was significantly associated with increased tumour size, high histological and invasion grades, lymph node metastasis and high mitotic index. Reduced β-catenin expression was associated with high histological and invasion grades. Anomalous expression of P-cadherin was only associated with invasion. In 39 cases for which follow-up data were available, reduced E-cadherin and β-catenin expression was significantly associated with shorter overall survival and disease free survival. Abnormal expression of adhesion molecules is a common phenomenon in canine mammary malignant tumours and may play a central role in tumour progression.

Keywords: Canine; Mammary tumours; E-cadherin; P-cadherin; β-Catenin

Introduction

Cadherins are calcium-dependent cell–cell adhesion molecules that play critical roles during embryogenesis and in the maintenance of normal adult tissue architecture (Takeichi, 1991; Gumbiner, 1996). Cadherins interact with several proteins termed catenins, including α-, β- and γ-catenin, which link cadherins to the actin cytoskeleton and mediate signal-transduction mechanisms that control cellular events, including cell polarity, differentiation, growth and migration (Knudsen et al., 1998).

The best characterised and most widely distributed members of the family are the classical cadherins, namely epithelial (E-) and placental (P-) cadherins (Nose and Takeichi, 1986). E-cadherin is found in almost all human epithelial tissues, whereas P-cadherin is restricted to the basal layers of stratified epithelium (Nose and Takeichi, 1986; Shimoyama et al., 1989). In normal human breast and canine mammary tissue, these molecules show a distinct pattern of expression; E-cadherin is expressed by luminal epithelial cells, whereas expression of P-cadherin is restricted to myoepithelial cells (Shimoyama et al., 1989; Palacios et al., 1995; Gama et al., 2002, 2004).

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Loss or down-regulation of E-cadherin/β-catenin complexes is associated with oncogenic progression in human breast cancer (Gamallo et al., 1993; Yoshida et al., 2001). In addition, anomalous epithelial expression of P-cadherin in human breast cancer is associated with aggressive biological behaviour and poor outcome (Palacios et al., 1995; Peralta Soler et al., 1999; Paredes et al., 2002, 2005).

The role of cadherins and catenins in canine mammary tumours is still poorly understood (Restucci et al., 1997; Reis et al., 2003; Brunetti et al., 2005; De Matos et al., 2007). Brunetti et al. (2005) showed that reduced E-cadherin/β-catenin expression influences invasion of canine mammary tumours, but not proliferation or survival. Loss of E-cadherin in canine mammary tumours was correlated with tumour size, ulceration, lymph node metastasis, necrosis and infiltrative growth (Matos et al., 2006). Recently, we found a significant association between P-cadherin expression in canine mammary tumours and histological type (Gama et al., 2004). In the present study, we correlated the expression of E- and P-cadherin and β-catenin in a series of malignant canine mammary tumours with clinicopathological parameters, proliferation and survival to study their possible role in canine mammary tumorigenesis.

Materials and methods

Source of tumours

Sixty-five malignant canine mammary tumours were selected from the histopathological files of the University of Trás-os-Montes and Alto Douro, Vila Real and from the Institute of Biomedical Science at the University of Porto, Portugal. The material had been fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (3 μm) were cut and stained with haematoxylin and eosin (HE) for histological examination and immunohistochemistry.

Case follow-up

Follow-up data were available for 39 cases for a mean of 15 months (range 1–36 months) after surgical treatment. Disease-free survival (DFS) and overall survival (OS) were calculated from the day of the surgery until the time of recurrence/metastasis or death, respectively. The cause of death was confirmed at post-mortem examination.

Histopathological examination

Tumours were classified according to the World Health Organization (WHO) criteria for canine mammary neoplasms (Misdorp et al., 1999) by three pathologists. Each tumour was assessed for size, skin ulceration, necrosis and mode of growth (expansile vs. infiltrative). Regional lymph nodes were available in 52 cases and assessed for the presence of metastases.

Malignant epithelial neoplasms were graded according to the Nottingham method for human breast tumours (Elston and Ellis, 1998). Tubule formation, nuclear pleomorphism and mitotic index were scored on a scale of 1–3 (slight, moderate or marked degree) and grades were based on the total score: grade I (well differentiated): 3–5 points; grade II (moderately differentiated): 6–7 points; and grade III (poorly differentiated): 8–9 points. Tumours were also graded for invasion according to Gilbertson et al. (1983): stage 0 (non-infiltrating); stage I (stromal invasion); and stage II (neoplastic emboli in vessels and/or lymph node involvement).

Immunohistochemistry

Monoclonal antibodies used in the present study were anti-E-cadherin (4A2C7, 1:100, Zymed Laboratory), anti-P-cadherin (clone 56, 1:50, BD Transduction Laboratories), anti-β-catenin (CAT-5H10, 1:100, Zymed Laboratory) and anti-Ki-67 (MIB-1, 1:50, Dakocytomation). A streptavidin–biotin-peroxidase complex method was used with a commercial detection system (Ultra Vision Detection System, Lab Vision Corporation) following the manufacturer's instructions.

Antigen retrieval for E-cadherin and β-catenin was carried out by microwave treatment in a 0.05% detergent solution (Extran, Merck) and for P-cadherin with ethylene diamine tetraacetic acid (EDTA) buffer pH 8.0 (Lab Vision Corporation) in a boiling water bath for 20 min. For Ki-67 antigen retrieval, slides were incubated with 0.2 mg/mL trypsin (Merck) in phosphate buffered saline (PBS) for 10 min at 37 °C prior to microwave treatment (3 × 5 min) in 10 mM citrate buffer, pH 6.0. Adjacent normal mammary tissues were used as internal positive controls. The primary antibody was replaced with PBS for negative controls.

Quantification of immunolabelling

P-cadherin expression in canine mammary tissues was assessed semi-quantitatively according to the percentage of immunoreactive cells (cells showing a membranous and/or cytoplasmic expression pattern) in negative (0: <10%) and positive (1: 10–25%; 2: 26–50%; 3: >50%) tumours (Gama et al., 2004). E-cadherin and β-catenin immunoreactivity was classified as membranous (localised at cell–cell boundaries) or cytoplasmic (uniformly distributed through the cytoplasm, with no recognisable distinction between membrane and cytoplasm). Nuclear expression of β-catenin was only detected in normal mammary gland epithelial cells adjacent to the tumour in two cases and therefore was not scored. Cases were grouped according to Brunetti et al. (2005) as "preserved", when positivity was membranous and higher than 75% of neoplastic epithelial cells and as "reduced" in all remaining samples, including negative tumours.

Combined variables were created for immunoreactivity patterns of E-cadherin/β-catenin (E-cad/β-cat), E-cadherin/P-cadherin (E-cad/P-cad) and β-catenin/P-cadherin (β-cat/P-cad) to investigate a possible relationship between any reduction/lack of expression of E-cadherin/β-catenin and over-expression of P-cadherin. Tumours were classified into four categories (+/+, +/−, −/+, −/−) for each combined variable.

Proliferative indices

Mitotic index and Ki-67 index were determined by counting 1000 neoplastic cells in the most mitotically active areas or areas with the highest Ki-67 positivity and were calculated as the percentage of tumour cells that exhibited mitotic figures or had positive staining for Ki-67, respectively.

Statistical analysis

For statistical analysis, associations between the expression of the different adhesion molecules and continuous variables (mitotic and Ki-67 indices) were assessed by analysis of variance (ANOVA). Associations between adhesion molecule expression and clinicopathological parameters (categorical variables), such as tumour size, histological type, histological grade and invasion, were performed using the χ^2 test. Fisher's exact test was performed when compared variables had exactly two groups (2 × 2 table), such as E-cadherin expression (preserved and reduced groups) versus ulceration (absent and present groups). Survival curves were generated by the Kaplan–Meyer method and the survival rates were compared using the log-rank test. All statistical analysis was performed using SPSS 11.5 statistical software. P values <0.05 were considered statistically significant.

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Results

The 65 malignant tumours examined in this study were classified as solid carcinomas (22 cases), tubulopapillary carcinomas (12), complex carcinomas (12), carcinosarcomas (12), spindle cell carcinomas (four) and sarcomas (three) according to WHO criteria. Epithelial tumours were classified as grade I (four cases), grade II (18) and grade III (40) according to the Nottingham method.

Expression of P-cadherin was evident in all histological types, whereas expression of E-cadherin and β-catenin was negative in all sarcomas. Reduction/lack of E-cadherin expression was evident in 27 malignant tumours (41.5%) and reduction/lack of β-catenin expression in 35 (53.8%). Aberrant cytoplasmic/membranous expression of P-cadherin was found in 56 (86.2%) malignant tumours. In 13 (20%) of these 56 tumours, 10–25% of neoplastic cells were positive; in 17 (26.2%), 26–50% were positive; and in 26 (40%) >50% were positive (Fig. 1).

Immunohistochemical expression of E-cadherin and β-catenin was significantly correlated ($P < 0.0001$): 26 cases were positive for both proteins, there was reduced expression of both proteins in 23 cases and 16 cases were discordant. We found no significant association between aberrant P-cadherin expression and reduced E-cadherin or β-catenin expression.

The relationship between cadherins and β-catenin expression and several clinicopathological variables is shown in Table 1. There was a significant difference across histological types in expression of E-cadherin ($P = 0.01$). Reduced E-cadherin expression was significantly related to infiltrative tumour growth ($P = 0.02$), higher histological grade ($P < 0.0001$), higher degree of invasion ($P = 0.002$) and lymph node metastasis ($P = 0.047$). Reduced β-catenin

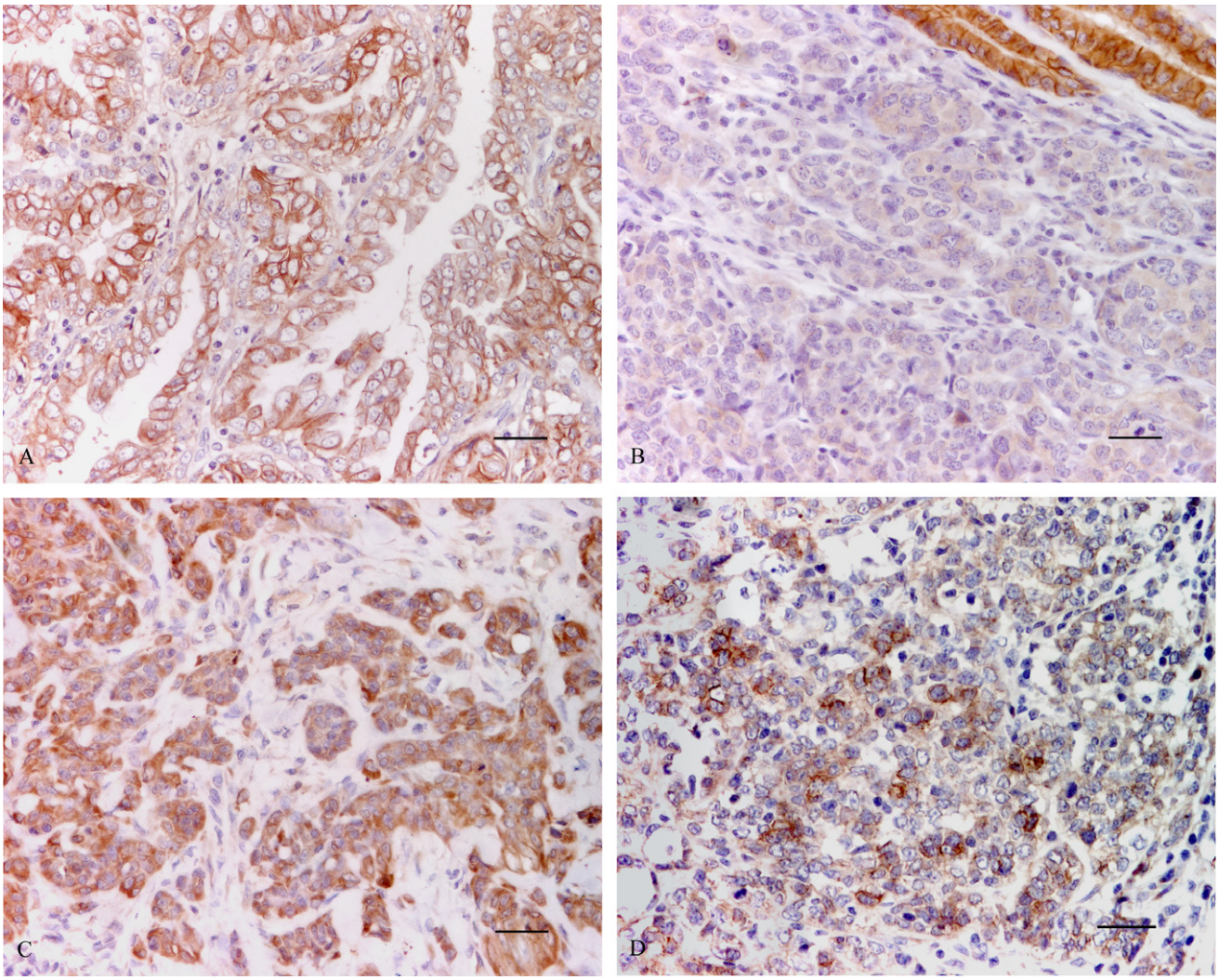

Fig. 1. Immunohistochemical expression of adhesion molecules in malignant canine mammary tumours. (A) Tubulopapillary carcinoma with preserved membranous E-cadherin expression. (B) Solid carcinoma. Neoplastic cells are negative for E-cadherin expression, whereas non-neoplastic epithelial cells are positive. (C) Solid carcinoma, with strong P-cadherin immunoreactivity. (D) Complex carcinoma showing reduced membranous β-catenin expression (Bar = 30 μm).

Table 1
Association between E-cadherin, P-cadherin and β-catenin expression and clinicopathological parameters

Clinicopathological parameter	*n*	E-cadherin		β-Catenin		P-cadherin			
		Pr[a]	Rd[b]	Pr	Rd	0	1	2	3
Tumour size[c]									
<3 cm	22	14 (63.6%)	8 (36.4%)	8 (36.4%)	14 (63.6%)	4 (18.2%)	5 (22.7%)	7 (31.8%)	6 (27.3%)
3–5 cm	26	16 (61.5%)	10 (38.5%)	16 (61.5%)	10 (38.5%)	3 (11.5%)	5 (19.2%)	7 (26.9%)	11 (42.3%)
>5 cm	16	7 (43.8%)	9 (56.3%)	5 (31.3%)	11 (68.8%)	2 (12.5%)	2 (12.5%)	3 (18.8%)	9 (56.3%)
P		0.43		0.1		0.77			
Ulceration									
Absent	55	32 (58.2%)	23 (41.8%)	26 (47.3%)	29 (52.7%)	7 (12.7%)	12 (21.8%)	15 (27.3%)	21 (38.2%)
Present	10	6 (60%)	4 (40%)	4 (40%)	6 (60%)	2 (20%)	1 (10%)	2 (20%)	5 (50%)
P		0.98		0.74		0.73			
Histological type									
Solid carcinoma	22	9 (40.9%)	13 (59.1%)	8 (36.4%)	14 (63.6%)	6 (27.3%)	6 (27.3%)	5 (22.7%)	5 (22.7%)
Tubulopapillary carcinoma	12	10 (83.3%)	2 (16.7%)	8 (66.7%)	4 (33.3%)	1 (8.3%)	2 (16.7%)	3 (25%)	6 (50%)
Complex carcinoma	12	10 (83.3%)	2 (16.7%)	6 (50%)	6 (50%)	1 (8.3%)	4 (33.3%)	3 (25%)	4 (33.3%)
Carcinosarcoma	12	6 (50%)	6 (50%)	6 (50%)	6 (50%)	0	1 (8.3%)	3 (25%)	8 (66.7%)
Spindle cell carcinoma	4	3 (75%)	1 (25%)	2 (50%)	2 (50%)	0	0	2 (50%)	2 (50%)
Sarcoma	3	0	3 (100%)	0	3 (100%)	1 (33.3%)	0	1 (33.3%)	1 (33.3%)
P		**0.01**		0.38		0.44			
Mode of growth									
Expansile	11	10 (90.9%)	1 (9.1%)	7 (63.6%)	4 (36.4%)	2 (18.2%)	5 (45.5%)	3 (27.3%)	1 (9.1%)
Infiltrative	54	28 (51.9%)	26 (48.1%)	23 (42.6%)	31 (57.4%)	7 (13%)	8 (14.8%)	14 (25.9%)	25 (46.3%)
P		**0.02**		0.32		**0.04**			
Necrosis									
Absent	6	5 (83.3%)	1 (16.7%)	4 (66.7%)	2 (33.3%)	0	1 (16.7%)	4 (66.7%)	1 (16.7%)
Present	59	33 (55.9%)	26 (44.1%)	26 (44.1%)	33 (55.9%)	9 (15.3%)	12 (20.3%)	13 (22%)	25 (42.4%)
P		0.38		0.40		0.1			
Histological grade[d]									
Grade I	4	4 (100%)	0	4 (100%)	0	1 (25%)	1 (25%)	1 (25%)	1 (25%)
Grade II	18	17 (94.4%)	1 (5.6%)	12 (66.7%)	6 (33.3%)	2 (11.1%)	4 (22.2%)	5 (27.8%)	7 (38.9%)
Grade III	40	17 (42.5%)	23 (57.5%)	14 (35%)	26 (65%)	6 (15%)	8 (20%)	10 (25%)	16 (40%)
P		**<0.0001**		**0.006**		0.99			
Invasion[e]									
Stage 0	11	11 (100%)	0	9 (81.8%)	2 (18.2%)	1 (9.1%)	5 (45.5%)	3 (27.3%)	2 (18.2%)
Stage I	11	8 (72.7%)	3 (27.3%)	5 (45.5%)	6 (54.5%)	1 (9.1%)	1 (9.1%)	5 (45.5%)	4 (36.4%)
Stage II	43	19 (44.2%)	24 (55.8%)	16 (37.2%)	27 (62.8%)	7 (16.3%)	7 (16.3%)	9 (20.9%)	20 (46.5%)
P		**0.002**		**0.03**		0.22			
Lymph node metastasis[f]									
Absent	27	20 (74.1%)	7 (25.9%)	13 (48.1%)	14 (5.9%)	3 (11.1%)	8 (29.6%)	9 (33.3%)	7 (25.9%)
Present	25	11 (44%)	14 (56%)	13 (52%)	12 (48%)	3 (12%)	3 (12%)	5 (20%)	14 (56%)
P		**0.047**		0.99		0.13			

Probability (*P*) values in bold are significant.
[a] Preserved.
[b] Reduced.
[c] Tumour size was available in 64 cases.
[d] According to the Nottingham method for human breast tumours (Elston and Ellis, 1998).
[e] According to Gilbertson et al. (1983).
[f] Lymph nodes were available in 52 cases.

expression was also associated with high histological grade ($P = 0.006$) and degree of invasion ($P = 0.03$). Anomalous expression of P-cadherin was associated only with invasion ($P = 0.04$).

When proliferation indices (mitotic index and Ki-67 index) were compared with expression patterns of the cadherins and β-catenin, there were significant differences only between higher mitotic counts and reduced expression of E-cadherin ($P = 0.03$) and β-catenin ($P = 0.003$) (Table 2). No significant differences were found between P-cadherin groups.

Tumours with reduced expression of E-cadherin and β-catenin were associated with significantly shorter survival times for both DFS (E-cadherin: $P = 0.0263$; β-catenin: $P = 0.0095$) and OS (E-cadherin: $P = 0.0245$; β-catenin: $P = 0.0113$) (Fig. 2). There was no significant association

Table 2
Association between E-cadherin, P-cadherin and β-catenin expression and proliferation indices

	n	Mitotic index	Ki-67 index
		Mean ± standard deviation	Mean ± standard deviation
E-cadherin			
Preserved	33	0.88 ± 0.58	24.98 ± 9.84
Reduced	27	1.27 ± 0.75	29.69 ± 9.31
P		**0.03**	0.064
β-Catenin			
Preserved	25	0.75 ± 0.49	24.54 ± 10.59
Reduced	35	1.27 ± 0.49	28.92 ± 8.93
P		**0.003**	0.088
P-cadherin			
<10%	8	1.45 ± 0.84	26.85 ± 8.75
10–25%	12	0.76 ± 0.42	24.1 ± 7.52
26–50%	15	1.02 ± 0.68	27.97 ± 9.93
>50%	25	1.09 ± 0.72	28.09 ± 11.18
P		0.179	0.693

Probability (*P*) values in bold are significant.

between increased expression of P-cadherin and DFS or OS (Fig. 3).

When tumours were grouped according to combined patterns of expression of E-cadherin/P-cadherin, β-catenin/P-cadherin and E-cadherin/β-catenin, there were significant differences in histological grade III ($P = 0.005$), invasion grades I and II ($P = 0.045$) and lymph node metastasis ($P = 0.029$) for E-cad/P-cad (−/+) cases, but no significant differences for E-cad/P-cad (+/−) or E-cad/P-cad (+/+) tumours (Supplementary Table 1). There was a significant association between β-cat/P-cad (−/+) immunoreactivity and less differentiated tumours ($P = 0.016$) when compared to other groups (Supplementary Table 2). Loss/reduction of immunoreactivity for E-cadherin, β-catenin, or both, was significantly associated with less differentiated tumours ($P = 0.003$), higher invasive grade ($P = 0.02$) and lymph node metastases ($P = 0.043$) (Supplementary Table 3). No statistical differences were found with the remaining variables.

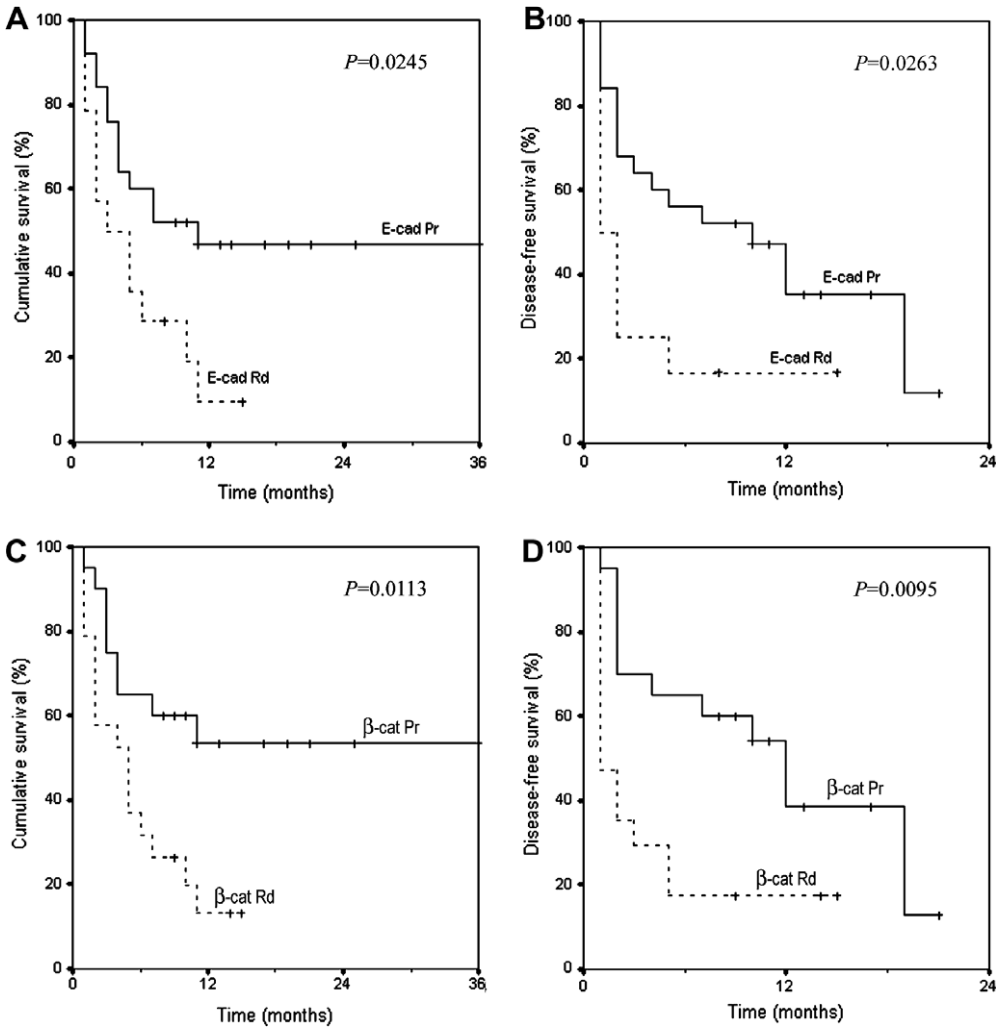

Fig. 2. Kaplan–Meier overall survival and disease-free survival curves of groups with preserved and reduced expression of E-cadherin (A, B) and β-catenin (C, D).

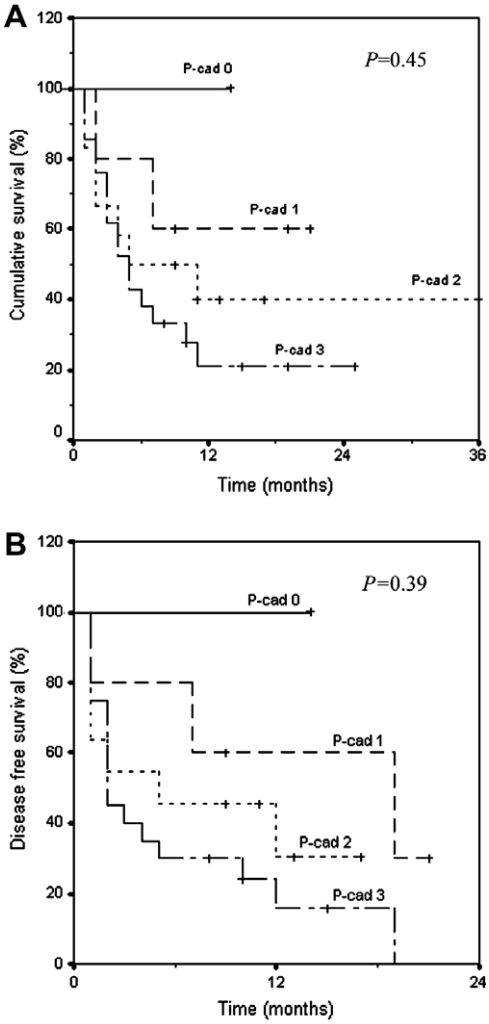

Fig. 3. Kaplan–Meier overall survival (A) and disease-free survival (B) curves of cases with <10% (0), 10–25% (1), 25–50% (2) or >50% (3) P-cadherin positive cells.

Discussion

Altered expression or absence of expression of the cadherin–catenin complex results in decreased adhesion of cells and has been implicated in tumorigenesis, particularly tumour cell invasion (Wijnhoven et al., 2000). Several previous reports have described the expression of cadherins and/or associated proteins in canine mammary gland tumours, but the patterns of expression of E-cadherin, P-cadherin and β-catenin have not been correlated with a wide range of clinicopathological features, including survival (Restucci et al., 1997; Reis et al., 2003; Gama et al., 2004; Brunetti et al., 2005; De Matos et al., 2007).

The present study demonstrated reduced membranous expression of E-cadherin and β-catenin in malignant canine mammary tumours compared to the normal mammary gland, suggesting that down-regulation of these molecules is a common event in canine mammary tumours. This finding is supported by other recent studies on canine mammary tumours (Brunetti et al., 2005; De Matos et al., 2007) and human breast cancer (Park et al., 2007). Although P-cadherin is not expressed in normal mammary epithelial cells, a subset of canine mammary tumours exhibited aberrant P-cadherin expression, as previously reported (Gama et al., 2004).

A relationship between E-cadherin and β-catenin expression was observed in canine mammary tumours in this study, corroborating previous reports of co-expression of these adhesion molecules in canine mammary tissue (Brunetti et al., 2005; De Matos et al., 2007) and human breast tissue (Gillet et al., 2001; Yoshida et al., 2001). This finding is consistent with the formation of adhesion complexes on the cell membrane. Some human breast cancer studies have reported an inverse correlation between loss of E-cadherin and aberrant P-cadherin immunoreactivity (Palacios et al., 1995; Peralta Soler et al., 1999; Gamallo et al., 2001), whereas we found no association between P-cadherin and other adhesion molecules.

In this study, reduced membranous expression of E-cadherin was significantly associated with histological type, poor differentiation, high invasiveness, high index of proliferation and lymph node metastasis. Previous studies on canine mammary tumours have made similar observations (Restucci et al., 1997; Reis et al., 2003; Brunetti et al., 2005; De Matos et al., 2007). Together with our results, this suggests a possible role for E-cadherin-mediated adhesion in preventing invasion and metastasis in canine mammary tumours, corroborating some studies in human breast cancer (Bankfalvi et al., 1999; Madhavan et al., 2001).

However, other studies on human breast cancer have not confirmed such a relationship (Palacios et al., 1995; Bukholm et al., 1998; Kovacs et al., 2003) or have associated preservation of expression of E-cadherin with lymph node metastasis (Gillet et al., 2001; Howard et al., 2005). In contrast to the present study, one previous report on canine mammary tumours showed no association between reduced E-cadherin expression and high histological grade (Matos et al., 2006). Our study identified an association between expression of E-cadherin and mitotic index, whereas no such association was identified by Brunetti et al. (2005). In human cancer studies, we also find opposing results for differentiation (Siitonen et al., 1996; Kovacs et al., 2003; Howard et al., 2005) and proliferation (Charpin et al., 1999; Fricke et al., 2003). Sample selection (histological type, stage, tumour grade), number of cases analysed and differences in staining evaluation may individually or in combination be held responsible for the observed discrepancies between different studies.

Reduced membranous β-catenin expression was found to be significantly associated with high grade and highly invasive tumours. These findings confirm a previous study (Brunetti et al., 2005) but contradict another (De Matos et al., 2007). In the present study, we found no association between the loss of β-catenin expression and the presence of lymph node metastases, which supports similar findings in canine (De Matos et al., 2007) and human studies (Bukholm et al., 1998; Gonzalez et al., 1999; Yoshida et al.,

2001), but we did observe a significant association between β-catenin reduction and high mitotic index, in contrast to a previous study in dogs (Brunetti et al., 2005).

The prognostic significance of E-cadherin and β-catenin expression in terms of survival of dogs with mammary carcinoma is unclear. Our data show that loss/reduction of E-cadherin and β-catenin expression is significantly associated with shorter OS and DFS, in contrast to previous findings by Brunetti et al. (2005). In human breast cancer studies, assessment of down-regulation of E-cadherin and catenins as prognostic markers of breast carcinoma has also proven problematic. Some authors claim that E-cadherin and β-catenin represent valuable prognostic markers (Siitonen et al., 1996; Dollet-Filhard et al., 2006; Park et al., 2007), whereas others have found no association between these molecules and survival time (Peralta Soler et al., 1999; Yoshida et al., 2001).

These conflicting results may reflect the current poor understanding of the dynamics of cell–cell adhesion. This process seems to be regulated at various levels, including gene transcription, protein stability and post-translational modification of the cadherin/catenin complex, in particular by phosphorylation of β-catenin. Besides its function in establishing tight cell adhesion, β-catenin plays a major role in cell signalling through interactions with receptor tyrosine kinases and transcription factors of the Lef/Tcf family, suggesting a dual role as a tumour suppressor and as an oncogene in human cancers (Wijnhoven et al., 2000).

Aberrant expression of P-cadherin was associated with invasion, but not with a higher invasion grade, which also takes into account vessel and/or lymph node involvement. Our results with canine mammary malignant tumours do not reflect some in vitro studies in human breast (Paredes et al., 2004) and pancreatic (Taniuchi et al., 2005) cancer cell lines, which suggest a proinvasive role for P-cadherin, through its interaction with signalling molecules such as $p120^{ctn}$ (Taniuchi et al., 2005).

In the present series, we did not find further associations between P-cadherin expressions and other clinicopathological variables or proliferation labelling indices. In our previous study, we described an association with tumour type (Gama et al., 2004), which was not confirmed in this larger series. In human cancer studies we also find contradictory results, probably related with sample selection. Paredes et al. (2005) found no significant correlation with histological type, although some authors suggested that P-cadherin was related with some special tumour types, such as medullary and metaplastic carcinomas (Palacios et al., 1995).

The present work supports our previous study, which did not find a statistically significant difference between P-cadherin aberrant expression and differentiation grade (Gama et al., 2004). However, in recent studies on human breast cancer, P-cadherin expression was significantly associated with increased histological grade (Palacios et al., 1995; Peralta Soler et al., 1999; Gamallo et al., 2001; Paredes et al., 2002, 2005; Kovacs et al., 2003). The small number of grade I tumours in our study may not have provided sufficient statistical power to establish an association.

Some human breast cancer studies (Palacios et al., 1995; Kovacs et al., 2003; Paredes et al., 2005) also failed to find a correlation between anomalous expression of P-cadherin and the presence of lymph node metastases. However, other studies have described an association with highly proliferative tumours (Paredes et al., 2005), lymph node metastases (Gamallo et al., 2001) and poor prognosis (Peralta Soler et al., 1999; Gamallo et al., 2001; Paredes et al., 2005). Although several authors suggested a possible role for P-cadherin in promoting aggressive tumour cell behaviour (Peralta Soler et al., 1999; Gamallo et al., 2001; Paredes et al., 2002, 2005), the biological significance of the anomalous P-cadherin in breast cancer is still poorly understood. As P-cadherin is expressed only by myoepithelial cells in normal breast tissue, the presence of this molecule might indicate a basal/myoepithelial differentiation (Peralta Soler et al., 1999), which has been associated with a poor outcome in human breast cancer (van de Rijn et al., 2002).

Despite some similarities with human breast cancer, canine mammary tumours are frequently associated with myoepithelial differentiation (Misdorp, 2002), which might explain the high percentage of P-cadherin positive tumours in our series. Although no correlation was found in the present study, we propose additional studies including a large series of simple carcinomas in order to investigate if P-cadherin expression is able to identify a subset of carcinomas with a particularly poor prognosis.

It is important to note that alterations in any component may lead to disrupted function of adhesion complexes. When we studied E-cadherin/β-catenin combinations, we found that the loss or reduction of at least one of these molecules was associated with less differentiated, highly invasive tumours, frequently with lymph node metastasis, supporting previous canine (Brunetti et al., 2005) and human studies (Bukholm et al., 1998; Wijnhoven et al., 2000). However, future studies are needed with a larger series and a longer follow up to investigate these interrelationships and correlate them with distinct tumour behaviours in the canine mammary gland.

Conclusions

The study has demonstrated altered expression of intercellular adhesion molecules in canine mammary malignant tumours. There were significant associations between reduced E-cadherin expression and some known prognostic parameters, such as tumour type, histological grade, invasiveness and lymph node metastasis and between reduced β-catenin expression and histological grade and invasiveness. Preserved expression of these molecules was associated with tumours of low mitotic index and with a better clinical outcome. We were also able to confirm aberrant P-cadherin expression in malignant mammary tumours,

which was associated with an infiltrative tumour growth, but with no relation to other clinicopathological variables.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tvjl.2007.05.024.

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Supplementary Table 1
Association between E-cad/P-cad combined expression and clinicopathological parameters.

Clinicopathological parameter	n	E-cad/P-cad			
		+/+	+/–	–/+	–/–
Tumour size[a]					
<3 cm	22	12 (54.5%)	2 (9.1%)	6 (27.3%)	2 (9.1%)
3-5 cm	26	15 (57.7%)	1 (3.8%)	8 (30.8%)	2 (7.7%)
>5 cm	16	6 (37.5%)	1 (6.3%)	8 (50%)	1 (6.3%)
P		0.83			
Ulceration					
Absent	55	29 (52.7%)	3 (5.5%)	19 (34.5%)	4 (7.3%)
Present	10	5 (50%)	1 (10%)	3 (30%)	1 (10%)
P		0.99			
Histological type					
Solid carcinoma	22	7 (31.8%)	2 (9.1%)	9 (40.9%)	4 (18.2%)
Tubulopapillary carcinoma	12	9 (75%)	1 (8.3%)	2 (16.7%)	0
Complex carcinoma	12	9 (75%)	1 (8.3%)	2 (16.7%)	0
Carcinosarcoma	12	6 (50%)	0	6 (50%)	0
Spindle cell carcinoma	4	3 (75%)	0	1 (25%)	0
Sarcoma	3	0	0	2 (66.7%)	1 (33.3%)
P		0.13			
Mode of growth					
Expansive	11	9 (81.8%)	1 (9.1%)	0	1 (9.1%)
Infiltrative	54	25 (46.3%)	3 (5.6%)	16 (40.7%)	4 (7.4%)
P		0.07			
Necrosis					
Absent	6	5 (83.3%)	0	1 (16.7%)	0
Present	59	29 (49.2%)	4 (6.8%)	21 (35.6%)	5 (8.5%)
P		0.34			
Histological grade[b]					
Grade I	4	3 (75%)	1 (25%)	0	0
Grade II	18	16 (88.9%)	1 (5.6%)	0	1 (5.6%)
Grade III	40	15 (37.5%)	2 (5%)	19 (47.5%)	4 (10%)
P		**0.005**			
Invasion[c]					
Stage 0	11	10 (90.9%)	1 (9.1%)	0	0
Stage I	11	7 (63.6%)	1 (9.1%)	3 (27.3%)	0
Stage II	43	17 (39.5%)	2 (4.7%)	19 (44.2%)	5 (11.6%)
P		**0.045**			
Lymph node metastasis[d]					
Absent	27	17 (63%)	3 (11.1%)	7 (25.9%)	0
Present	25	11 (44%)	0	11 (44%)	3 (12%)
P		**0.029**			

[a] Tumour size was available in 64 cases; [b] According to the Nottingham method for human breast tumours (Elston and Ellis, 1998); [c] According to Gilbertson *et al*. (1983); [d] Lymph nodes were available in 52 cases

Supplementary Table 2
Association between β-cat/P-cad combined expression and clinicopathological parameters.

Clinicopathological parameter	n	β-cat/P-cad			
		+/+	+/–	–/+	–/–
Tumour size[a]					
<3 cm	22	8 (36.4%)	0	10 (45.5%)	4 (18.2%)
3-5 cm	26	14 (53.8%)	3 (11.5%)	8 (30.8%)	1 (14.3%)
>5 cm	16	5 (31.3%)	0	9 (56.3%)	2 (12.5%)
P		0.13			
Ulceration					
Absent	55	24 (43.6%)	3 (5.5%)	23 (41.8%)	5 (9.1%)
Present	10	4 (40%)	0	4 (40%)	2 (20%)
P		0.76			
Histological type					
Solid carcinoma	22	7 (31.8%)	1 (4.5%)	9 (40.9%)	5 (22.7%)
Tubulopapillary carcinoma	12	7 (58.3%)	1 (8.3%)	4 (33.3%)	0
Complex carcinoma	12	6 (50%)	0	5 (41.7%)	1 (8.3%)
Carcinosarcoma	12	6 (50%)	0	6 (50%)	0
Spindle cell carcinoma	4	2 (50%)	0	2 (50%)	0
Sarcoma	3	0	1 (33.3%)	1 (33.3%)	1 (33.3%)
P		0.26			
Mode of growth					
Expansive	11	7 (63.6%)	0	2 (18.2%)	2 (18.2%)
Infiltrative	54	21 (38.9%)	3 (5.6%)	25 (46.3%)	5 (9.3%)
P		0.23			
Necrosis					
Absent	6	4 (66.7%)	0	2 (33.3%)	0
Present	59	24 (40.7%)	3 (5.1%)	25 (42.4%)	7 (11.9%)
P		0.69			
Histological grade[b]					
Grade I	4	3 (75%)	1 (25%)	0	0
Grade II	18	12 (66.7%)	0	4 (22.2%)	2 (11.1%)
Grade III	40	13 (32.5%)	1 (2.5%)	21 (52.5%)	5 (12.5%)
P		**0.016**			
Invasion[c]					
Stage 0	11	9 (81.8%)	0	1 (9.1%)	1 (9.1%)
Stage I	11	4 (36.4%)	1 (9.1%)	6 (54.5%)	0
Stage II	43	15 (34.9%)	2 (4.7%)	20 (46.5%)	6 (14%)
P		0.095			
Lymph node metastasis[d]					
Absent	27	12 (44.4%)	1 (3.7%)	12 (44.4%)	2 (7.4%)
Present	25	12 (48%)	2 (8%)	9 (42.9%)	2 (8%)
P		0.89			

[a] Tumour size was available in 64 cases; [b] According to the Nottingham method for human breast tumours (Elston and Ellis, 1998); [c] According to Gilbertson *et al*. (1983); [d] Lymph nodes were available in 52 cases

Supplementary Table 3
Association between E-cad/β-cat combined expression and clinicopathological parameters.

Clinicopathological parameter	n	E-cad/β-cat			
		+/+	+/–	–/+	–/–
Tumour size[a]					
<3 cm	22	8 (36.4%)	6 (27.3%)	0	8 (36.4%)
3-5 cm	26	13 (50%)	3 (11.5%)	3 (11.5%)	7 (26.9%)
>5 cm	16	4 (25%)	3 (18.8%)	1 (6.3%)	8 (50%)
P		0.31			
Ulceration					
Absent	55	22 (40%)	10 (18.2%)	4 (7.3%)	19 (34.5%)
Present	10	4 (40%)	2 (20%)	0	4 (40%)
P		0.92			
Histological type					
Solid carcinoma	22	5 (22.7%)	4 (18.2%)	3 (13.6%)	10 (45.5%)
Tubulopapillary carcinoma	12	8 (66.7%)	2 (16.7%)	0	2 (16.7%)
Complex carcinoma	12	6 (50%)	4 (33.3%)	0	2 (16.7%)
Carcinosarcoma	12	5 (41.7%)	1 (8.3%)	1 (8.3%)	5 (41.7%)
Spindle cell carcinoma	4	2 (50%)	1 (25%)	0	1 (25%)
Sarcoma	3	0	0	0	3 (100%)
P		0.23			
Mode of growth					
Expansile	11	7 (63.6%)	3 (27.3%)	0	1 (9.1%)
Infiltrative	54	19 (35.2%)	9 (16.7%)	4 (7.4%)	22 (40.7%)
P		0.11			
Necrosis					
Absent	6	4 (66.7%)	1 (16.7%)	0	1 (16.7%)
Present	59	22 (37.3%)	11 (18.6%)	4 (6.8%)	22 (37.3%)
P		0.56			
Histological grade[b]					
Grade I	4	4 (100%)	0	0	0
Grade II	18	12 (66.7%)	5 (27.8%)	0	1 (5.6%)
Grade III	40	10 (25%)	7 (17.5%)	4 (10%)	19 (47.5%)
P		**0.003**			
Invasion[c]					
Stage 0	11	9 (81.8%)	2 (18.2%)	0	0
Stage I	11	5 (45.5%)	3 (27.3%)	0	3 (27.3%)
Stage II	43	12 (27.9%)	7 (16.3%)	4 (9.3%)	20 (46.5%)
P		**0.02**			
Lymph node metastasis[d]					
Absent	27	13 (48.1%)	7 (25.9%)	0	7 (25.9%)
Present	25	9 (42.9%)	2 (8%)	4 (16%)	10 (40%)
P		**0.043**			

[a] Tumour size was available in 64 cases. [b] According to the Nottingham method for human breast tumours (Elston and Ellis, 1998); [c] According to Gilbertson *et al*. (1983); [d] Lymph nodes were available in 52 cases.

Chapter IV

Immunohistochemical expression of Epidermal Growth Factor Receptor (EGFR) in canine mammary tissues.

Gama A, Gärtner F, Alves A, Schmitt F (submitted)

Immunohistochemical expression of Epidermal Growth Factor Receptor (EGFR) in canine mammary tissues

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Abstract

Epidermal Growth Factor Receptor (EGFR) has been extensively studied in human breast cancer; however, systematic studies of EGFR protein expression in canine mammary gland tumours are lacking. Therefore, we evaluated its immunohistochemical expression in a series of 136 canine mammary tumours and representative areas of adjacent normal and hyperplastic mammary tissue and investigated a possible correlation between EGFR overexpression and several clinicopathological parameters and survival. In normal and hyperplastic canine mammary glands, EGFR expression was consistently observed in myoepithelial cells, with luminal cells usually negative. In tumour tissues, EGFR overexpression was found in 9 benign (19.6%) and 38 malignant (42.2%) lesions, with EGFR positivity significantly related with malignancy. Besides animal age and tumour size, there were no significant associations between other clinicopathological parameters and EGFR overexpression. On survival analysis, tumours with EGFR overexpression showed a reduced disease-free and overall survival; however these associations failed to reach statistically significant levels.

Keywords: canine; mammary tumours; EGFR; immunohistochemistry

Introduction

Epidermal Growth Factor Receptor-1 (EGFR) is a member of the human epidermal growth factor receptor (HER) family. This family includes four closely related tyrosine kinase receptors (EGFR or erbB1, HER-2/neu or erbB2, HER-3 or erbB3, and HER-4 or erbB4) and has been receiving great attention in recent human literature (Hynes and Lane, 2005; Park *et al*., 2007; Sassen *et al*., 2008).

EGFR overexpression has been found in 16-48% of human breast cancer, generally associated with poor clinical outcome and aggressive biological properties (Sainsbury *et al*., 1985; Lewis *et al*., 1990; Klijn *et al*., 1992; Toi *et al*., 1994; Tsutsui *et al*., 2002). The interest in EGFR is further enhanced by the availability and US Food and Drug Administration approval of specific EGFR tyrosine kinase inhibitors, which are currently being tested in human patients with lung and breast cancer (Baselga and Arteaga, 2005).

Mammary gland tumors are the most commonly occurring neoplasm in the female dog (Nerurkar *et al*., 1989; Misdorp, 2002) but despite their high incidence and clinical importance, a few number of studies are available with respect to EGFR. In addition, although EGFR has been identified by several methodologies in canine mammary gland tissues such as radioligand binding or immunoenzymatic assays, to the best of our knowledge there are currently no data addressing the analysis of EGFR expression by immunohistochemistry. Therefore, the aim of the present study was to assess EGFR immunohistochemical expression in normal, benign and malignant canine mammary gland tissues and correlate its expression with clinicopathological parameters and survival, in order to provide some information on its biological significance and potential diagnostic use.

Material and methods

Tumour specimens

A hundred and thirty six cases of canine benign (n=46) and malignant (n=90) mammary tumours were selected from the histopathological files of the University of Trás-os-Montes and Alto Douro, Vila Real, and from the Institute of Biomedical Science of the

University of Porto, Portugal. The material was fixed in 10% neutral formalin and embedded in paraffin wax. Sections (3 μm) were cut and stained with haematoxylin and eosin (HE) for histological examination, or used to perform immunohistochemistry.

Sixty four malignant tumour cases had available follow up data. Dogs were followed after surgical treatment, with a mean follow-up period of 13 months (range, 5-74 months). Disease-free survival (DFS) and overall survival (OS) were calculated from the day of the surgery until the time of recurrence/metastasis or death, respectively.

Histological Examination

Tumours were diagnosed according to the World Health Organization criteria for canine mammary neoplasms (Misdorp *et al*., 1999). Clinicopathological variables included in the present study were: age, breed, presence of ovariohysterectomy, contraceptive administration, tumour size, tumour location, presence of skin ulceration, tumour histological type, presence of intra-tumoral necrosis, mode of growth (expansive vs. infiltrative), presence of stromal and vascular invasion and presence of lymph node metastasis.

Malignant tumours were also graded histologically in accordance with the Nottingham method for human breast tumours (Elston and Ellis, 1998), based on the assessment of three morphological features: tubule formation, nuclear pleomorphism and mitotic counts. Each of these features was scored on a scale of 1 to 3 to indicate whether it was present in slight, moderate or marked degree, giving a putative total of 3-9 points. Grade was allocated by an arbitrary division of the total points as follows: grade I (well differentiated), 3, 4 or 5 points; grade II (moderately differentiated), 6 or 7 points; and grade III (poorly differentiated), 8 or 9 points.

Immunohistochemistry

Immunohistochemistry was performed with a mouse monoclonal antibody raised against EGFR (Clone 31G7, 1:50, Zymed Laboratories, San Francisco, California, USA). Antigen retrieval was carried out by enzyme digestion: sections were incubated with 0.4% pepsin (Dako, Denmark) in HCl 0.01 N solution (pH=2) for 30 minutes at 37ºC. Slides were incubated overnight with EGFR antibody in a humid chamber at 4ºC.

A polymeric labelling methodology was used as a detection system (Novolink Polymer Detection System, Novocastra, Newcastle, United Kingdom), following the manufacturer's instructions. Adjacent normal mammary tissues were used as internal positive controls. Negative controls were carried out by replacing the primary antibody with PBS.

Quantification of Immunolabelling

To evaluate EGFR expression in canine mammary tissues Herceptest scoring system was applied (0=no membrane staining or <10% of cells stained; 1+=incomplete membrane staining in >10% of cells; 2+=>10% of cells with weak to moderate complete membrane staining; and 3+=strong and complete membrane staining in >10% of cells) (Reis Filho *et al*., 2005), with 2+ and 3+ cases considered positive.

Statistical Analysis

To compare variables of interest χ^2 and Fisher's exact test (two-sided) were used when appropriate. The Kaplan-Meier method was used to examine OS and DFS curves, and comparisons of these curves were assessed by using the log-rank test. Analysis was performed by SPSS 11.5 software statistics and two values were considered significant when $P<0.05$.

Results

In this study, the 46 benign tumours examined were classified as: benign mixed tumours (22 cases), complex adenomas (13 cases) and basaloid adenomas (11 cases) and the 90 malignant tumours were classified as: complex carcinomas (30 cases), solid carcinomas (21 cases), carcinosarcomas (13 cases), tubulopapillary carcinomas (12 cases), carcinoma in benign tumours (7 cases), spindle cell carcinomas (4 cases) and anaplastic carcinomas (3 cases). According to the Nottingham method, malignant tumours were classified as grade I (17 cases), grade II (30 cases) and grade III (43 cases).

In normal and hyperplastic canine mammary ducts and lobules, a strong EGFR expression was consistently observed in the myoepithelial cell layer. Luminal epithelial

cells were usually negative, with the exception of some normal and hyperplastic ducts showing EGFR expression in both epithelial layers, results which are in accordance to previous human studies (Santini *et al*., 2002). The surrounding stroma was usually EGFR negative; however, perilobular stroma was commonly positive.

In benign tumours, EGFR expression was observed in both epithelial cell components; however, luminal epithelial cells usually showed a reduced level of expression (score 0 and 1+). On the other hand, the malignant tumours analysed showed a significant higher proportion of EGFR epithelial expression (Fig. 1). In fact, a complete membrane EGFR immunostaining was observed in more than 10 % of neoplastic cells (score 2+ and 3+) in 38 out of 90 (42.2%) malignant tumours, versus 9 out of 46 (19.6%) benign lesions.

The relationship between EGFR immunoexpression and clinicopathological parameters are shown in Tables 1 and 2, respectively. Benign and malignant lesions differed significantly (P=0.013) when considering EGFR expression. It was also found a significant association between EGFR expression and animal age (P=0.028) and tumour size (P=0.013). There was no significant association with all other tested variables ($P>0.05$).

Malignant tumours with EGFR overexpression (score 2+ and 3+) were usually related with shorter survival times for both disease free survival and overall survival. However, despite the differences observed, these associations failed to reach statistically significant levels (Disease free survival: P=0.08; Overall survival: P=0.09) (Fig. 2).

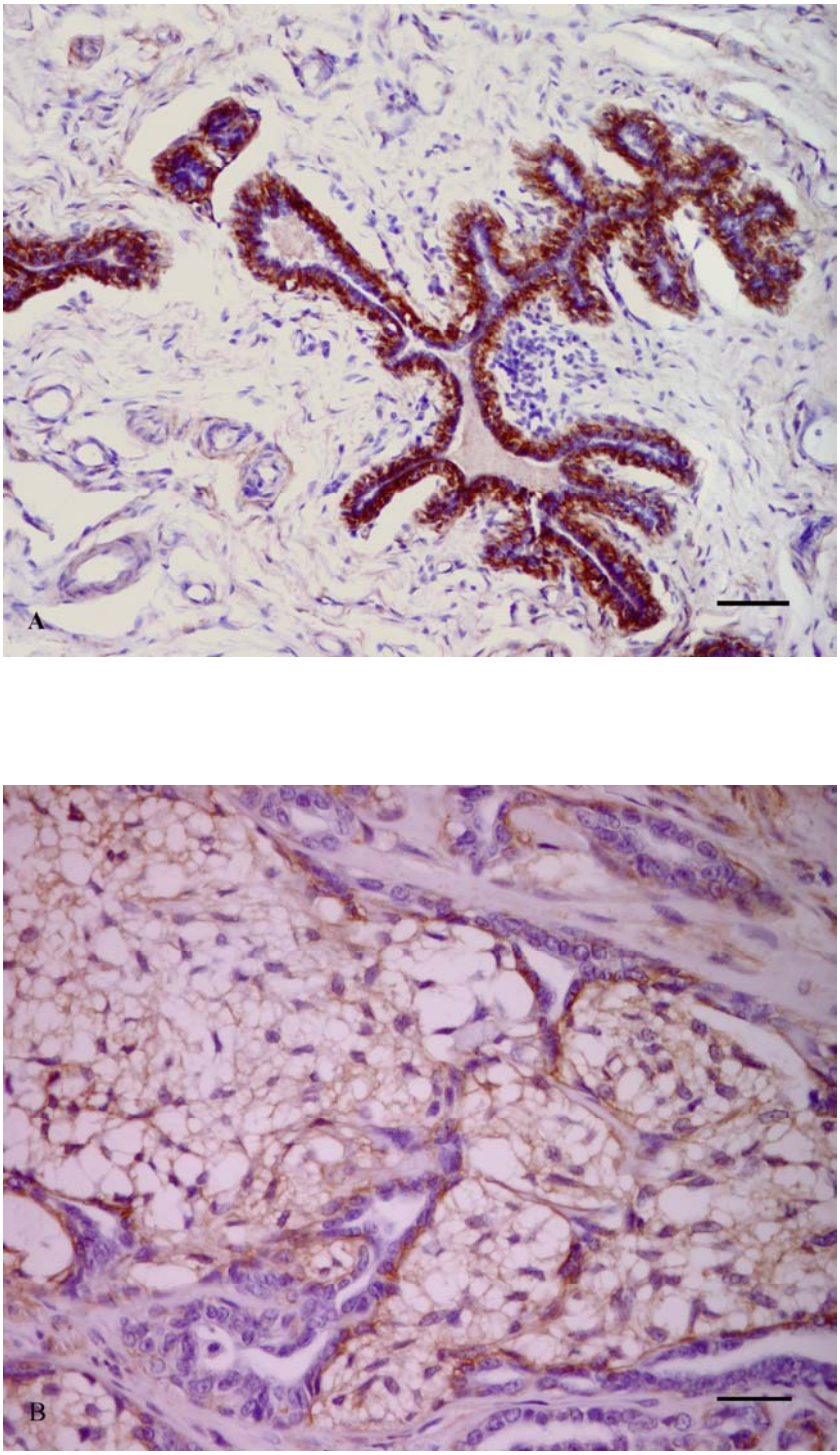
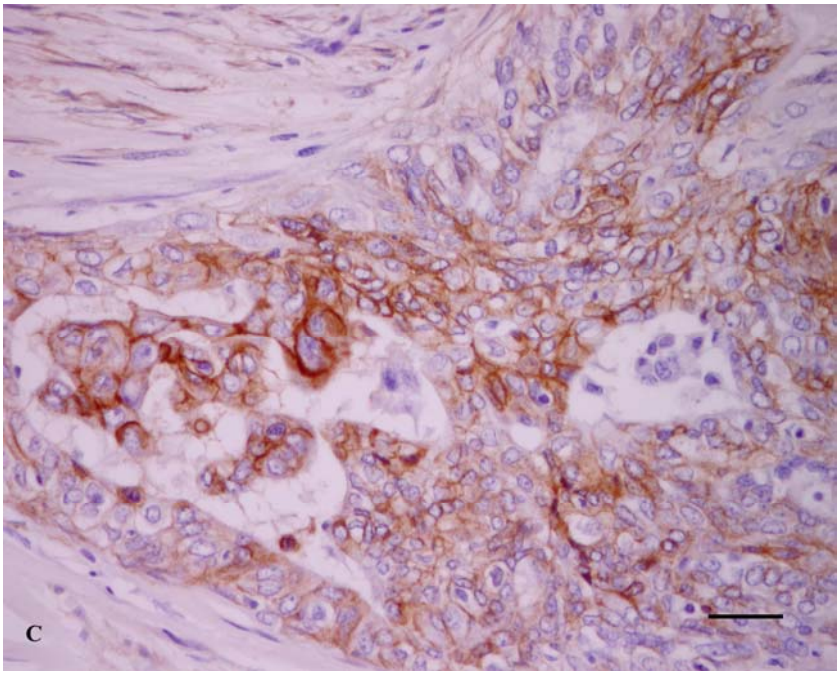


Fig. 1. Immunohistochemical expression of EGFR in canine mammary tissues. A. Normal mammary gland showing EGFR immunoexpression in the myoepithelial cell layer. Epithelial cells were negative; bar=60 μm; B. Complex adenoma with myoepithelial spindle and stellate-shaped cells showing EGFR expression. Epithelial cells are unreactive (0 score); bar=30 μm.



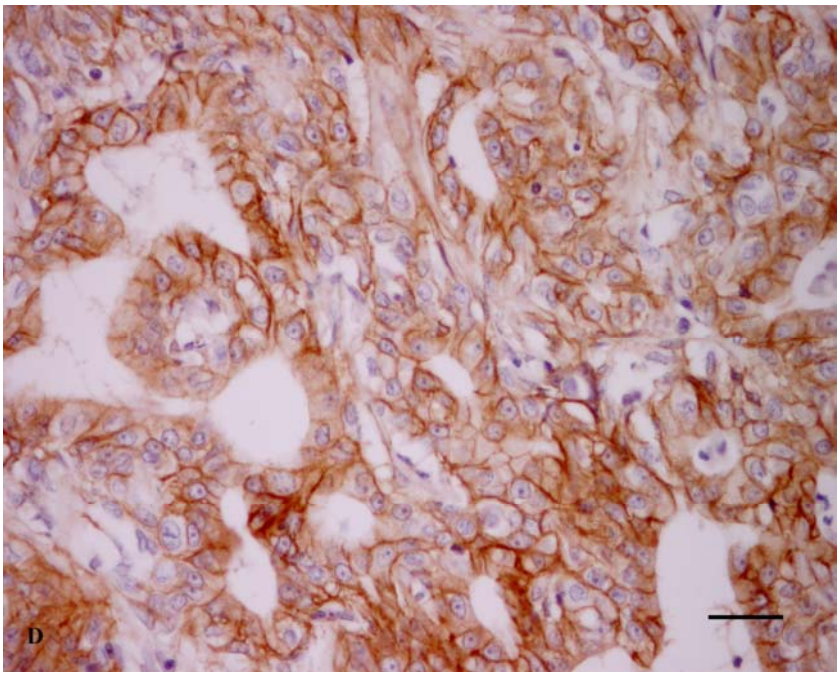


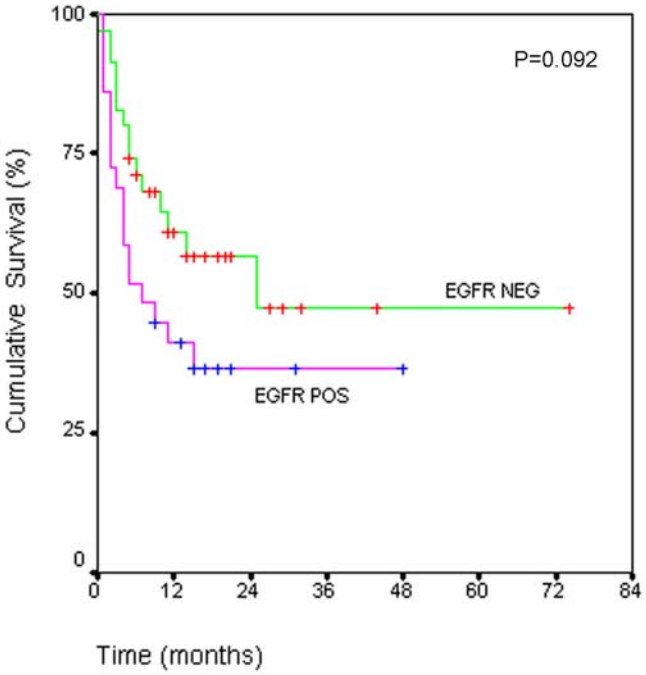
Fig. 1. (cont.) Immunohistochemical expression of EGFR in canine mammary tissues. C. Complex carcinoma, with epithelial neoplastic cells EGFR positive (2+ score); D. Tubulopapillary carcinoma showing epithelial neoplastic cells strongly positive for EGFR (3+ score); bar=30 μm.

Table 1: Association between EGFR expression and clinical parameters.

Clinical parameters	n	EGFR	
		Negative	Positive
Age			
≤ 9 years old	69	51 (73.9%)	18 (26.1%)
> 9 years old	60	33 (55%)	27 (45%)
P		**0.028**	
Breed			
Mixed breed	42	27 (64.3%)	15 (35.7%)
Poodle	25	19 (76%)	6 (24%)
Cocker spaniel	15	11 (73.3%)	4 (26.7%)
Boxer	6	5 (83.3%)	1 (16.7%)
Labrador retriever	6	4 (66.7%)	2 (33.3%)
Others	34	19 (55.9%)	15 (44.1%)
P		0.588	
Tumour size			
<3 cm	74	57 (77%)	17 (23%)
3-5 cm	33	19 (57.6%)	14 (42.4%)
>5 cm	25	12 (48%)	13 (52%)
P		**0.013**	
Tumour location			
Cranial glands (1 and 2)	6	4 (66.7%)	2 (33.3%)
Medial gland (3)	9	5 (55.6%)	4 (44.4%)
Caudal glands (4 and 5)	45	30 (66.7%)	15 (33.3%)
Multiple	15	8 (53.3%)	7 (46.7%)
P		0.837	
Skin ulceration			
Absent	120	81 (67.5%)	39 (32.5%)
Present	13	6 (46.2%)	7 (53.8%)
P		0.137	
Ovariohysterectomy			
No	60	34 (56.7%)	26 (43.3%)
Yes, prior to tumour development	12	8 (66.7%)	4 (33.3%)
Yes, performed with mastectomy	14	10 (71.4%)	4 (28.6%)
P		0.554	
Contraception			
No	62	39 (62.9%)	23 (37.1%)
Yes	8	4 (50%)	4 (50%)
P		0.702	

Table 2: Association between EGFR expression and pathological parameters.

Pathological parameters	n	EGFR	
		Negative	Positive
Histological diagnoses			
Benign lesions	46	37 (80.4%)	9 (19.6%)
Malignant lesions	90	52 (57.8%)	38 (42.2%)
P		**0.013**	
Histological type			
Benign mixed tumour	22	18 (81.8%)	4 (18.2%)
Complex adenoma	13	10 (76.9%)	3 (23.1%)
Basaloid adenoma	11	9 (81.8%)	2 (18.2%)
Solid carcinoma	21	14 (66.7%)	7 (33.3%)
Tubulopapillary carcinoma	12	6 (50%)	6 (50%)
Complex carcinoma	30	16 (53.3%)	14 (46.7%)
Carcinosarcoma	13	9 (69.2%)	4 (30.8%)
Spindle cell carcinoma	4	1 (25%)	3 (75%)
Carcinoma in benign tumour	7	5 (71.4%)	2 (28.6%)
Anaplastic carcinoma	3	1 (33.3%)	2 (66.7%)
P		0.192	
Necrosis			
Absent	39	29 (74.4%)	10 (25.6%)
Present	97	60 (61.9%)	37 (38.1%)
P		0.231	
Mode of growth			
Expansive	75	52 (69.3%)	23 (30.7%)
Infiltrative	61	37 (60.7%)	24 (39.3%)
P		0.365	
Histological grade			
Grade I	17	10 (58.8%)	7 (41.2%)
Grade II	30	16 (53.3%)	14 (46.7%)
Grade III	43	26 (60.5%)	17 (39.5%)
P		0.85	
Stromal Invasion			
Absent	24	12 (50%)	12 (50%)
Present	65	39 (60%)	26 (40%)
P		0.472	
Lymphovascular Invasion			
Absent	41	22 (53.7%)	19 (46.3%)
Present	47	29 (61.7%)	18 (38.3%)
P		0.519	
Lymph node metastasis			
Absent	23	12 (52.2%)	11 (47.8%)
Present	23	13 (56.5%)	10 (43.5%)
P		0.99	



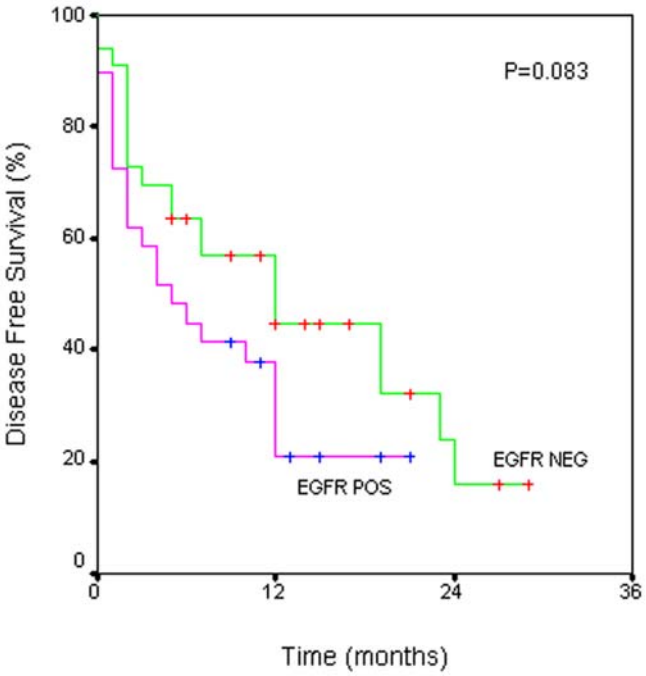


Fig. 2. Kaplan-Meier overall survival (A) and disease-free survival (B) curves of groups with negative (0 and 1+ scores) and positive (2+ and 3+ scores) EGFR expression.

Discussion

EGFR was the first tyrosine kinase transmembrane receptor to be directly linked with human cancer (Hynes and Lane, 2005). EGFR is a member of the human epidermal growth factor receptor family that consists of an extracellular domain binding EGF or TGF-α, a short transmembrane domain and an intracellular domain carrying tyrosine kinase activity (Carpenter and Cohen, 1979). EGFR pathway contributes to several processes involved in tumour survival and growth, including cell proliferation/differentiation, angiogenesis and metastasis, which make this molecule an attractive target for cancer prevention and treatment (Shien *et al*., 2005).

While the expression of EGFR gene has been extensively investigated in human breast cancer (Klijn *et al*., 1992; Bhargava *et al*., 2005; Reis Filho *et al*., 2005; Reis-Filho *et al*., 2006; Park *et al*., 2007), there are only a limited number of reports available in canine literature demonstrating its presence in mammary gland tissues (Nerurkar *et al*., 1987; Donnay *et al*., 1993; Rutteman *et al*., 1994; Donnay *et al*., 1996; Matsuyama *et al*., 2001). These previous reports describe EGFR concentration or expression in normal and tumorous canine mammary gland based on distinct methodologies, with a lack of information about its immunohistochemical pattern of cellular distribution and on the possible correlation between EGFR expression and a wide range of clinicopathological features, including survival.

The present study demonstrates that myoepithelial cells in normal, hyperplastic and benign lesions constantly express EGFR, similarly to previous studies in human breast (Moller *et al*., 1989; Santini *et al*., 2002). Thus, alike human breast tissues, EGFR immunoreactivity appears to be of diagnostic use for myoepithelial cell identification in canine mammary gland, in addition to specific immunohistochemical markers such as p63, P-cadherin, α-smooth muscle actin, CK14 or calponin (Destexhe *et al*., 1993; Espinosa de Los Monteros *et al*., 2002; Gama *et al*., 2003; Gama *et al*., 2004). According to several authors, EGFR myoepithelial expression in human breast tissues can be related to the recently recognized paracrine function by which myoepithelial cells exert their mechanical and functional barrier role in the juxtaposition between epithelium and stroma (Moller *et al*., 1989; Santini *et al*., 2002).

Nevertheless, despite our and previous demonstration of EGFR presence in the normal canine mammary gland (Donnay *et al*., 1993; Rutteman *et al*., 1994; Donnay *et al*., 1996), the research performed by Matsuyama and coworkers (2001) failed to find EGFR mRNA in normal canine mammary tissues, discrepancy that might be related with the different techniques used in each study.

In the present series, EGFR overexpression (2+ and 3+ scores) was found in 9/46 (19.6%) benign and 38/90 (42.2%) malignant tumours, with its expression significantly related with malignancy (P=0.013). These results are in contradiction with previous reports, which found no significant differences on EGFR concentration between normal, benign and malignant canine mammary tissues (Nerurkar *et al*., 1987; Rutteman *et al*., 1994; Donnay *et al*., 1996). However, because the methods used in those studies were different from ours, a direct comparison is difficult.

EGFR overexpression was only found to be associated with old aged animals and large sized tumours. No association was observed with other clinicopathological parameters, which is at some point in accordance to Donnay *et al.* (1993), who described no significant differences between EGFR concentrations and the clinicopathological parameters evaluated, including animal age, tumour location and histology. Similarly, there are contradictory results in human literature with respect to EGFR relationship with known prognostic factors (Fox *et al*., 1994; Toi *et al*., 1994; Pirinen *et al*., 1995; Reis-Filho *et al*., 2006).

The underlying mechanisms of EGFR protein overexpression are not completely understood. Gene amplification has been recently described in human breast carcinomas and *EGFR* activating mutations were also found, although uncommon (Al-Kuraya *et al*., 2004; Bhargava *et al*., 2005; Reis-Filho *et al*., 2005; Reis-Filho *et al*., 2006). It is likely that, in the majority of cases, EGFR up-regulation happens at the transcriptional level (Kersting *et al*., 2004). Given that this gene is consistently expressed in normal myoepithelial cells (Santini *et al*., 2002) and in human breast tumours with basal and/or myoepithelial differentiation (Nielsen *et al*., 2004; Shien *et al*., 2005; Reis-Filho *et al*., 2006), some authors argue that EGFR overexpression would constitute the maintenance of a myoepithelial phenotype or would be part of a transcriptomic programme of myoepithelial/"basal-like" differentiation (Reis-Filho *et al*., 2005). Canine mammary gland tumours are frequently associated with myoepithelial differentiation (Misdorp,

2002), and this fact is probably related with the relatively high percentage of EGFR positive tumours in our series.

EGFR is a recent tumour marker whose prognostic value has been well studied in humans, but still remains controversial (Klijn *et al*., 1992). Although some studies involving immunohistochemical analysis of EGFR expression in human breast tumours have shown a significant correlation of EGFR positivity with a shorter disease-free and overall survival (Tsutsui *et al*., 2002), others did not found such a correlation, and no consensus has been reached on the role of EGFR as a prognostic indicator (Ciardiello and Tortora, 2003).

In the present series, there was no association between EGFR overexpression and DFS or OS, whereas there was a tendency toward shorter DFS and OS in dogs with positive EGFR expression. In order to make definitive conclusions about the relationships and prognostic value of EGFR status in canine mammary tumours, larger studies with long term follow-up periods are warranted. In addition, studies designed to unravel the mechanism associated with EGFR overexpression in canine malignant tumours are also justified, considering that EGFR tyrosine kinase inhibitors might be used as potential therapeutic agents.

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Chapter V

Expression and prognostic significance of CK19 in canine malignant mammary tumours

Gama A, Alves A, Schmitt F (submitted)

Expression and prognostic significance of CK19 in canine malignant mammary tumours

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Abstract

The prognostic significance of the expression of cytokeratins (CK) in canine mammary malignant tumours is still unclear. We have examined the expression pattern of the luminal cytokeratin CK19 in a series of 102 canine mammary carcinomas by immunohistochemical analysis, and associated its expression with known prognostic markers, proliferation and survival. We also compared CK19 with the presence of basal/myoepithelial cell markers. Reduced/absent CK19 was significantly associated with histological type, invasiveness, high histological grade and an elevated Ki-67 index. CK19 positive expression was significantly associated with the presence of ER, whereas its reduced immunostaining was associated with basal/myoepithelial cell markers positive expression. Survival analysis demonstrated that down-regulation of this luminal CK is significantly associated with shorter overall and disease-free survival rates; however CK19 was not an independent prognostic factor in multivariate analysis. Thus, CK19 down-regulation in canine mammary carcinomas is related to an aggressive phenotype and seems to play a role in tumour progression.

Keywords: Canine; Mammary tumours; Cytokeratin 19; Prognosis

Introduction

Mammary gland tumours are the most commonly occurring neoplasm in the female dog and are known for their biological and histomorphological heterogeneity (Ferguson, 1985; Nerurkar *et al*, 1989; Moulton, 1990). Despite several immunohistochemical studies on the diagnostic value of specific luminal and basal/myoepithelial cell markers in canine mammary tumours (Hellmén and Lindgren, 1989; Griffey *et al*., 1993; Vos *et al*., 1993a, b; Espinosa de los Monteros, 2002; Gama *et al*., 2003, 2004), the increasing prevalence of canine neoplasia compels the veterinary pathologist not only to determine a precise diagnosis but also to assess prognosis (Zaidan Dagli, 2008).

Cytokeratin (CK) is one of the three types of intermediate filaments that constitute the cytoskeleton of epithelial mammalian cells (Moll *et al*., 1982; Chu and Weiss, 2002). CKs comprise a family of related proteins encoded by different genes, which are expressed in various epithelial cells in a developmentally regulated and differentiation-dependent manner (Romano *et al*., 1988; Bocker *et al*., 2002; Chu and Weiss, 2002). Simple epithelia express cytokeratins of low molecular weight, whereas stratified epithelia contain high molecular weight keratins.

In the normal human breast, the majority of luminal cells express CK7, CK8, CK18 and CK19, while basal/myoepithelial cells express CK5, CK14, CK15 and CK17 (Bocker *et al*., 2002). Considering that all of these cells can undergo malignant change, breast carcinomas can be classified as expressing a luminal or a basal phenotype (Sorlie *et al*., 2001; Abd El-Rehim *et al*., 2004), with several studies describing a significant association between basal phenotype and poor prognosis (van de Rijn *et al*., 2002; Abd El-Rehim *et al*., 2004).

As for the canine species, we and others have described the expression of a number of cell differentiation markers, such as CK (Hellmén and Lindgren, 1989; Destexhe *et al*., 1993; Griffey *et al*., 1993; Vos *et al*., 1993a, b and c; Rabanal and Else, 1994), p63 (Gama *et al*., 2003; Ramalho *et al*., 2004), P-cadherin (Gama *et al*., 2004, 2007); SMA (Destexhe *et al*., 1993; Vos *et al*., 1993a, b and c) and calponin (Espinosa de los Monteros *et al*., 2002) in mammary gland tissues. CK 8, 18 and 19 have been considered luminal cell markers (Griffey *et al*., 1993; Vos *et al*., 1993a, b and c; Rabanal and Else, 1994), whereas CK5, 14 and 17 (Griffey *et al*., 1993; Vos *et al*., 1993a, b and c), p63 and P-cadherin (Gama *et al*., 2003, 2004, 2007) were found to be expressed by basal/myoepithelial cells. SMA and calponin were exclusively expressed

by myoepithelial cells (Destexhe *et al*., 1993; Vos *et al*., 1993a, b and c; Espinosa de los Monteros *et al*., 2002). Based on the immunohistochemical expression of CK14, Griffey *et al*. (1993) applied the "basal carcinoma" nomenclature to canine carcinomas. Similarly to humans, these carcinomas showed aggressive clinical behaviour (Griffey *et al*., 1993) but, to the best of our knowledge, no investigations have been performed associating CK expression and prognosis in canine mammary tumours.

Recently, down-regulation of luminal CKs 18 and 19 has been identified as a significant predictor of aggressive disease in breast cancer patients (Woelfle *et al*., 2004; Parikh *et al*., 2008). We sought to evaluate the immunohistochemical expression of the CK19 luminal cell marker in a series of malignant canine mammary tumours and its possible correlation with some relevant clinicopathological parameters, namely proliferation, oestrogen receptor status, other cell-specific cell markers (basal/myoepithelial) and survival, in order to investigate its possible value as a prognostic marker in canine mammary cancer.

Material and methods

Tumour specimens

The present study is based on a series of a 102 cases of canine malignant mammary tumours selected from the histopathological files of the University of Trás-os-Montes and Alto Douro, Vila Real, and from the Institute of Biomedical Science at the University of Porto, Portugal. The material was fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (3 μm) were cut and stained with haematoxylin and eosin (HE) for histological examination or immunohistochemistry.

Case follow-up

Sixty nine dogs (n = 69) were followed post-surgically by the referring surgeons and presented a median overall survival time of 15 months (range 5-74 months). Overall survival (OS) was defined as the period between surgery and animal natural death or euthanasia due to cancer. Disease-free survival (DFS) was defined as the period of time between surgery and recurrent or metastatic disease. During the follow-up period, according to the referring surgeons, 35 animals died or were euthanized due to

metastatic disease and/or local recurrence. One dog died due to causes unrelated to the mammary tumour and it was censored at the time of death (19 months).

Histological Examination

Tumours were diagnosed according to the World Health Organization (WHO) criteria for canine mammary neoplasms (Misdorp *et al*., 1999). Clinicopathological variables included in the present study were: age, breed, ovariohysterectomy status, contraceptive administration, tumour size, tumour location, presence of skin ulceration, tumour histological type, presence of intra-tumoral necrosis, mode of growth (expansive vs. infiltrative), presence of stromal and vascular invasion and presence of lymph node metastasis.

Tumours were evaluated for grade in accordance with the Nottingham method for human breast tumours (Elston and Ellis, 1998), based on the assessment of three morphological features: tubule formation, nuclear pleomorphism and mitotic counts. Each of these features was scored on a scale of 1-3 to indicate whether it was present in slight, moderate or marked degree, giving a putative total of 3-9 points. Grade was allocated by an arbitrary division of the total points as follows: grade I (well differentiated): 3-5 points; grade II (moderately differentiated): 6-7 points; and grade III (poorly differentiated): 8-9 points.

Immunohistochemistry

Tissue sections were incubated with primary monoclonal antibodies against CK19, Ki-67, oestrogen receptor (ER), CK5, CK14, calponin, p63, P-cadherin and α-smooth muscle actin (SMA). Table 1 summarises the antibodies used and the staining procedures adopted for each antibody. Antigen retrieval was carried out by microwave treatment in 10 mM citrate buffer, pH 6.0, with the exception of P-cadherin, which was treated with an ethylene diamine tetraacetic acid (EDTA) buffer, pH 8.0 (LabVision) in a boiling bath for 20 min. For Ki-67 antigen retrieval, slides were incubated with 0.2 mg/mL trypsin (Merck) in phosphate buffered saline (PBS) for 10 min at 37 ºC. After cooling (20 min at room temperature), the sections were immersed in 3% hydrogen peroxide and distilled water for 30 min to block endogenous peroxidase activity. All slides were incubated with a blocking serum (LabVision) for 10 min and then incubated with the specific antibody.

After incubation, slides were incubated with biotinylated secondary antibody, followed by streptavidin-conjugated peroxidase (LabVision), except for ER. For this antibody, a polymeric labelling methodology was used as a detection system (Novolink Polymer Detection System, Novocastra), following the manufacturer's instructions. Subsequently, the colour was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) and slides were counterstained with Gill's haematoxylin, dehydrated, and mounted for evaluation by light microscopy.

Adjacent normal mammary tissues were used as internal positive controls. Negative controls were carried out by replacing the primary antibody with PBS.

Table 1. Primary monoclonal antibodies and immunostaining protocols used

Antibody	Origin	Clone	Dilution	Pretreatment	Incubation
CK19	Neomarkers, USA	BA17	1:150	Microwave	Overnight
Ki-67	Dako, Denmark	Mib1	1:50	Trypsin + Microwave	Overnight
ER	Novocastra, UK	NCL-LH2	1:40	Microwave	2 hours
CK5	Neomarkers, USA	XM26	1:25	Microwave	Overnight
CK14	Novocastra, UK	NCL-LL002	1:20	Microwave	2 hours
CALP	Dako, Denmark	CALP	1:400	Microwave	2 hours
P63	Neomarkers, USA	4A4	1:150	Microwave	Overnight
PCAD	BD Transduction, USA	56	1:50	Water bath, 98ºC	Overnight
SMA	Novocastra, UK	NCL-SMA	1:50	Microwave	2 hours

Evaluation of the immunohistochemical data

CK19 positivity was indicated by the presence of cytoplasmic staining in neoplastic cells. For the evaluation of CK19 expression, we adopted a scoring method based on the estimation of the staining intensity in combination with an estimation of the percentage of immunoreactive cells (Parikh *et al*., 2008). As for staining intensity, CK19 expression was scored as: 0: no staining; 1: weak to moderate intensity; and 2: strong intensity. As for percentage, tumours were evaluated in <10% positive cells and ≥10% positive cells. For statistical purposes, CK19 status was considered positive if a tumour presented ≥10% positive epithelial cells, with a moderate or strong immunoreactivity.

Ki-67 immunostaining was evaluated as described previously (Gama *et al*., 2008). Immunoreactivities were classified by estimating the percentage of tumour cells showing characteristic staining. Nuclear ER immunoreactivity was considered positive when more than 10% of the neoplastic cells expressed this marker. A semi-quantitative analysis was performed for calponin, CK5, CK14, SMA and p63: 0: <10% positive

cells; 1: 10-50% positive cells; and 2: >50% positive cells, with a cytoplasmic (calponin, CK5, CK14, SMA) or nuclear (p63) pattern of cellular distribution. P-cadherin immunostaining was evaluated semi-quantitatively as previously described (Gama *et al*., 2004, 2008): 0: <10% positive cells; 1: 10-25% positive cells; 2: 26-50% positive cells and 3: >50% positive cells, with cells showing a membranous and/or cytoplasmic expression pattern.

Statistical Analysis

Associations between expression of CK19 and continuous variables (mitotic and Ki-67 indices) were assessed by the non parametric Mann-Whitney test. Associations between CK19 expression and categorical variables, such as tumour size, histological type, histological grade and invasion, were performed using the χ^2 test. Fisher's exact test was performed when compared variables had exactly two groups (2 x 2 table). Survival curves were generated by the Kaplan-Meier method and the survival rates were compared using the log-rank test. The combined effects of CK19 with previously recognised prognostically relevant variables were examined via Cox proportional hazards model. All statistical analysis was performed using SPSS 11.5 statistical software. A *P* value <0.05 was considered to be statistically significant.

Results

Patients and tumour characteristics

The mean age of dogs at the time of surgical removal of tumours was 9.6 ± 2.4 years (range 4-16 years). Most dogs in our series were mixed breeds (n = 34, 34.7%), followed by Toy poodles (n = 18, 18.4%) and Cocker spaniels (n = 11, 11.2%). The mean maximum tumour diameter was 4.24 ± 3.4 cm (range 0.5-18 cm), with tumours more frequently located in caudal mammary glands (n = 39; 57.4%). Skin ulceration was present in 19 cases (19%). In 11/74 (14.9%) female dogs with available clinical information, ovariohysterectomy was performed prior to the removal of mammary tumours. There was a history of contraceptive administration in 8/63 (12.7%) cases. Histologically, tumour types comprised 45/102 (44.1%) simple carcinomas, 45/102

(44.1%) complex carcinomas and 12/102 (11.8%) carcinosarcomas. According to the Nottingham method, tumours were classified as grade I (n = 17, 16.7%), grade II (n = 34, 33.3%) and grade III (n = 51, 50%).

CK19 expression in canine mammary malignant tumours

CK19 was consistently expressed in the normal adjacent canine mammary gland, being exclusively observed in luminal epithelial cells (Fig. 1A). In contrast, CK19 expression was variable in malignant tumour tissues. Cytoplasmic expression was evident in more than 10% of epithelial cells in 78/102 (76.5%) malignant tumours, whereas 24/102 (23.5%) presented with no staining or staining in less than 10% neoplastic cells (Fig. 1B and C).

The relationships between CK19 status and several clinicopathological variables are shown in Tables 2 and 3. The proportion of CK19 positive and negative tumours differed significantly with gland location ($P = 0.04$), histological type ($P = 0.002$), tumour growth pattern ($P = 0.001$), histological grade ($P<0.0001$) and stromal and lymphovascular invasion ($P = 0.003$ and $P<0.0001$, respectively), with most CK19 negative tumours exhibiting aggressive phenotypical features, such as high histological grade and stromal/vascular invasion.

The associations found between CK19 expression and the presence of other molecular cell markers are summarised in Table 4. With regard to these cell markers, CK19 expression showed significant differences across the different staining groups for ER, CK5, P-cadherin, p63 and calponin. Eighteen out of 22 (81.8%) CK19 negative tumours were ER negative ($P<0.0001$), 15/24 (62.5%) CK19 negative tumours were CK5 positive ($P = 0.015$), 19/23 (82.6%) CK19 negative tumours were P-cadherin positive ($P = 0.039$), 21/24 (87.5%) CK19 negative tumours were p63 positive and 13/24 (54.2%) CK19 negative tumours were calponin positive. No significant differences were observed between CK19 expression and CK14 or SMA staining patterns.

As shown in Table 4, compared with positive tumours, the CK19 negative group of tumours showed higher mitotic and Ki-67 labelling indices, being significantly associated with Ki-67 proliferative index ($P = 0.017$).

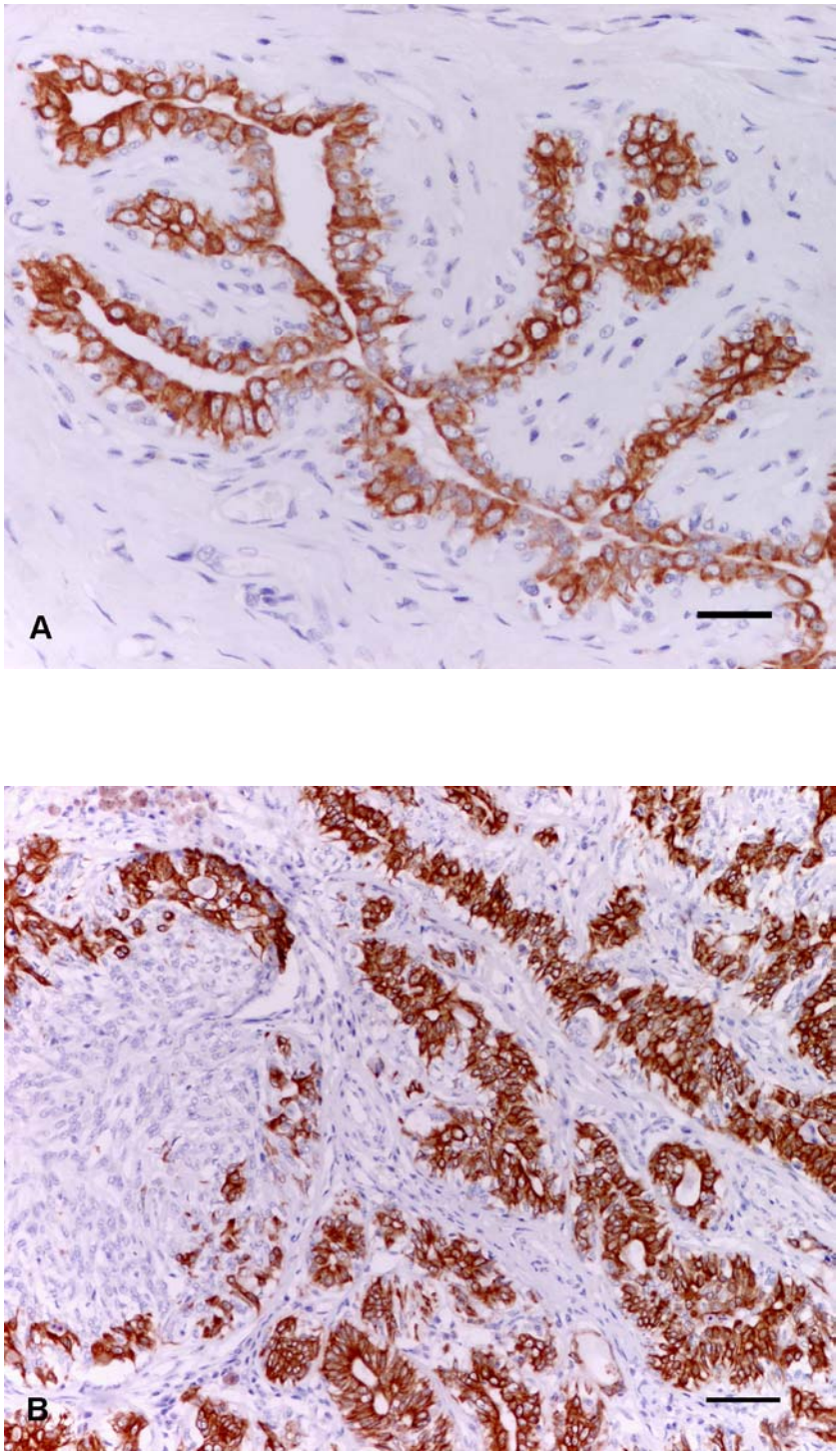

Fig. 1. Immunohistochemical expression of CK19 in canine mammary tissues. A. Normal mammary duct, showing CK19 expression restricted to luminal epithelial cells. Bar = 30 μm. B. Complex carcinoma (grade II) with strong cytoplasmic CK19 expression in more than 10% of epithelial cells. Bar = 60 μm.

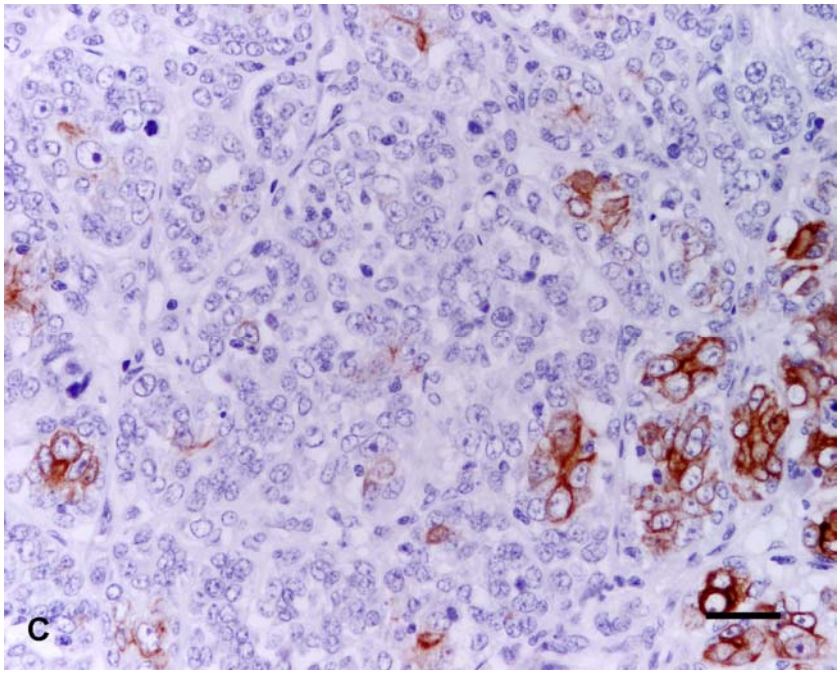

Fig. 1. (cont.) Immunohistochemical expression of CK19 in canine mammary tissues. C. Solid carcinoma (grade III) negative for CK19 immunostaining, with less than 10% positive neoplastic cells. Bar = 30 μm.

Prognostic significance of CK19 expression

Follow up data is summarised in Table 5. Kaplan-Meier survival curves revealed that a down-regulated expression of CK19 was significantly associated with lower overall (P = 0.0001, Fig. 2A) and disease-free (P = 0.0004, Fig. 2B) survival. To investigate whether CK19 expression represents an independent prognostic factor for canine mammary cancer, a multivariate analysis, including the histopathological parameters lymph node status and grade of differentiation, was performed; however the data showed that CK19 expression was not an independent prognostic parameter.

Table 2. Association between CK19 expression and clinical parameters

Clinical parameters	n	CK19 expression	
		Negative	Positive
Age	97[a]		
≤ 9 years old	47	10 (21.3%)	37 (78.7%)
> 9 years old	50	12 (24%)	38 (76%)
P		0.81	
Breed	98[a]		
Mixed breed	34	9 (26.5%)	25 (73.5%)
Poodle	18	5 (27.8%)	13 (72.2%)
Cocker spaniel	11	2 (18.2%)	9 (81.8%)
Others	35	7 (20%)	28 (80%)
P		0.87	
Tumour size	95[a]		
<3 cm	36	6 (16.7%)	30 (83.3%)
3-5 cm	33	10 (30.3%)	23 (69.7%)
>5 cm	26	7 (26.9%)	19 (73.1%)
P		0.42	
Tumour location	68[a]		
Cranial glands (1 and 2)	5	2 (40%)	3 (60%)
Medial gland (3)	10	1 (10%)	9 (90%)
Caudal glands (4 and 5)	39	13 (33.3%)	26 (66.7%)
Multiple	14	0 (0%)	14 (100%)
P		**0.04**	
Skin ulceration	100[a]		
Absent	81	18 (22.2%)	63 (77.8%)
Present	19	4 (21.1%)	15 (78.9%)
P		0.99	
Ovariohysterectomy	74[a]		
No	51	14 (27.5%)	37 (72.5%)
Yes, prior to tumour development	11	0 (0%)	11 (100%)
Yes, performed with mastectomy	12	4 (33.3%)	8 (66.7%)
P		0.13	
Contraception	63[a]		
No	55	13 (23.6%)	42 (76.4%)
Yes	8	2 (25%)	6 (75%)
P		0.99	

[a] Total number of cases for which clinical information was available

Table 3. Association between CK19 expression and pathological parameters

Pathological parameters	n	CK19	
		Negative	Positive
Histological type			
Simple carcinoma	45	17 (37.8%)	28 (62.2%)
Complex carcinoma	45	3 (6.7%)	42 (93.3%)
Carcinosarcoma	12	4 (33.3%)	8 (66.7%)
P		**0.002**	
Necrosis			
Absent	5	0 (0%)	5 (100%)
Present	97	24 (24.7%)	73 (75.3%)
P		0.58	
Mode of growth			
Expansive	31	1 (3.2%)	30 (96.8%)
Infiltrative	71	23 (32.4%)	48 (67.6%)
P		**0.001**	
Histological grade			
Grade I	17	0 (0%)	17 (100%)
Grade II	34	1 (2.9%)	33 (97.1%)
Grade III	51	23 (45.1%)	28 (54.9%)
P		**<0.0001**	
Stromal invasion			
Absent	27	1 (3.7%)	26 (96.3%)
Present	75	23 (30.7%)	52 (69.3%)
P		**0.003**	
Lymphovascular invasion			
Absent	43	2 (4.7%)	41 (95.3%)
Present	59	22 (37.3%)	37 (62.7%)
P		**<0.0001**	
Lymph node metastasis[a]			
Absent	24	4 (16.7%)	20 (83.3%)
Present	30	7 (23.3%)	23 (76.7%)
P		0.73	

[a] Lymph nodes were available in 54 cases

Table 4. Association between CK19 expression and other molecular markers and proliferation indices

Molecular markers[a, b]	n	CK19	
		Negative	Positive
ER			
Negative	40	18 (45%)	22 (55%)
Positive	56	4 (7.1%)	52 (92.9%)
P		**<0.0001**	
CK5			
0	20	9 (45%)	11 (55%)
1	49	6 (12.2%)	43 (87.8%)
2	33	9 (27.3%)	24 (72.7%)
P		**0.015**	
CK14			
0	17	5 (29.4%)	12 (70.6%)
1	56	12 (21.4%)	44 (78.6%)
2	29	7 (24.1%)	22 (75.9%)
P		0.77	
P-cadherin			
0	9	1 (11.1%)	8 (88.9%)
1	23	2 (8.7%)	21 (93.3%)
2	22	4 (18.2%)	18 (81.8%)
3	42	16 (38.1%)	26 (61.9%)
P		**0.039**	
P63			
0	29	3 (10.3%)	26 (89.7%)
1	40	4 (10%)	36 (90%)
2	33	17 (51.5%)	16 (48.5%)
P		**<0.0001**	
Calponin			
0	37	11 (29.7%)	26 (70.3%)
1	47	4 (8.5%)	43 (91.5%)
2	18	9 (50%)	9 (50%)
P		**0.001**	
SMA			
0	54	13 (24.1%)	41 (75.9%)
1	35	5 (14.3%)	30 (85.7%)
2	11	5 (45.5%)	6 (54.5%)
P		0.09	
Median Mitotic index (Min-Max)		0.98 (0.1-1.9)	0.59 (0-2.99)
P		0.05	
Median Ki-67 index (Min-Max)		27.97 (12.10-39.40)	21.06 (5.39-56.36)
P		**0.017**	

[a] Immunohistochemical evaluation of ER and P-cadherin was available in 96 cases; SMA was available in 100 cases and Ki-67 was available in 95 cases; [b] Score for CK5, CK14, SMA and p63: 0: <10% positive cells; 1: 10-50% positive cells; and 2: >50% positive cells; score for P-cadherin: 0: <10% positive cells; 1: 10-25% positive cells; 2: 26-50% positive cells and 3: >50% positive cells.

Table 5. Survival rates in dogs with available follow up

		Overall survival			Disease-free survival	
CK19 status	n[a]	Mean survival (months)	Average 1 year survival rate n (%)	n	Mean survival (months)	Average 1 year survival rate n (%)
Negative	14	5	1 (9.5)	14	4	0 (0.0)
Positive	55	43	30 (62.9)	54	13	19 (41.6)
P		0.0001			0.0004	

[a] Follow up data was available in 69 cases for OS and 68 cases for DFS.

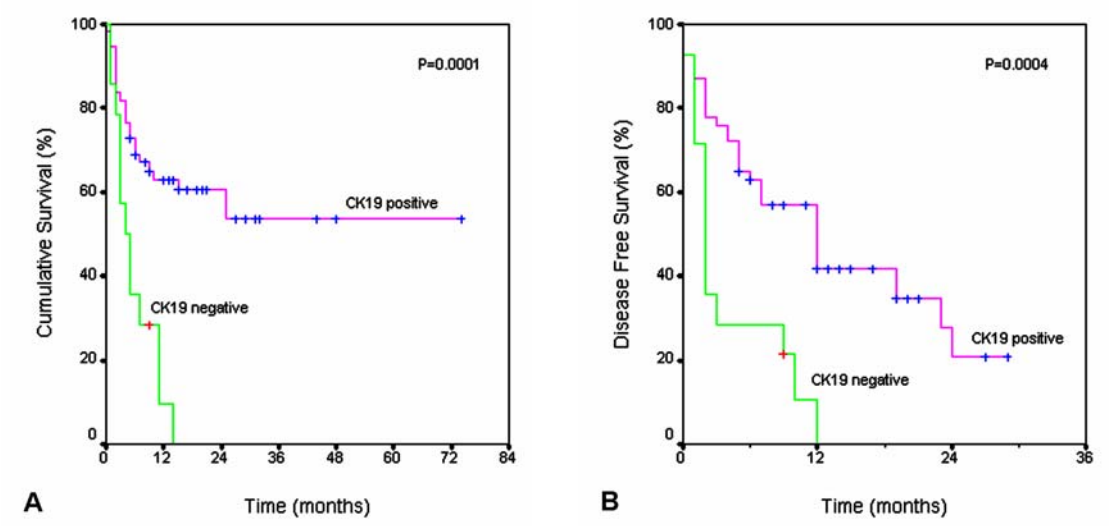

Fig. 2. Kaplan-Meier overall survival (A) and disease-free survival (B) curves of groups with positive and negative CK19 expression (*P*=0.0001 and *P*=0.0004, respectively).

Discussion

About half of canine mammary tumours are considered to be malignant and it is of great importance to recognise and identify reliable prognostic factors to estimate the individual risk of an unfavourable clinical outcome (Misdorp, 2002; Zaidan Dagli, 2008). Down-regulation of CK19 has been identified as an independent prognostic factor in human breast cancer (Parikh *et al*., 2008) and the present study demonstrates that CK19 might have a putative role in canine mammary tumour progression although it is not an independent prognostic parameter.

CKs have been recognised as epithelial cell markers in diagnostic histopathology for over 20 years (Moll *et al*., 1982; Coulombe and Omary, 2002). In the human mammary gland, immunohistochemistry has demonstrated that luminal cells usually express CK8,

18, and 19, while basal/myoepithelial cells express CK5, CK14, CK15 and CK17 (Moll *et al*., 1982; Bocker *et al*., 2002). CK19 expression also has been described in canine mammary tissues as a luminal cell marker (Destexhe *et al*., 1993; Vos *et al*., 1993c; Sarli *et al*., 2007). To the best of our knowledge, this is the first study correlating its expression pattern with clinicopathological parameters and clinical outcome for canine mammary carcinomas.

Immunohistochemical studies have shown that human breast carcinomas usually retain the CK pattern of normal luminal epithelial cells (Moll *et al*., 1982). Monoclonal antibodies directed against luminal CK18 and 19 have been used to identify primary and metastatic human breast cancer cells (Malzahn *et al*., 1998). A subset of tumours has been identified with down-regulation of luminal CKs associated with aggressive biological behaviour and poor outcome (Woelfle *et al*., 2004; Parikh *et al*., 2008), challenging the view that CKs are purely marker proteins.

Moreover, a number of regulatory changes in CK expression at the transcriptional and post-transcriptional level have been described in experimental cell studies (Blouin *et al*., 1992; Choi *et al*., 2000). Regulatory mechanisms include interaction with keratin associated proteins (KAPs), which results in CK phosphorylation, glycosylation, transglycosylation, caspase cleavage and ubiquitination (Coulombe and Omary, 2002). CKs may also associate with other cytoskeletal elements (Coulombe and Omary, 2002). Additional evidence for a more widespread role of CKs has come from mouse gene knockout studies, in which the double deletion of CK18 and 19 results in the lack of a functional CK skeleton and causes embryonic lethality (Hesse *et al*., 2000).

Our immunohistochemical results demonstrated a variable CK19 expression among canine mammary malignant tumours, with down-regulation of CK19 in 23.5% of tumours. Similar observations have already been described in the literature on canine mammary tumours (Destexhe *et al*., 1993; Vos at al., 1993b). With regard to clinicopathological parameters, most CK19 negative tumours were associated with aggressive phenotypical features, such as high histological grade and stromal/vascular invasion and high Ki-67 (proliferation) index. These results are in accordance with several reports in the human literature for luminal CKs markers, which found that loss of luminal CKs was significantly associated with a higher tumour grade and a higher mitotic index (Schaller *et al*., 1996; Abd El-Rehim *et al*., 2004; Woelfle *et al*., 2004; Willipinski-Stapelfeldt *et al*., 2005; Parikh *et al*., 2008).

Recent studies using gene array technology on human breast tumours have identified distinct subtypes of breast carcinomas (luminal A, luminal B, Her2 over-expressing and basal-like) that are associated with different clinical outcomes (Perou *et al*., 2000; Sorlie *et al*., 2001; Sorlie *et al*., 2003). Luminal A and B subtypes are based on the expression of ER, usually with CK19 expression (Birnbaum *et al*., 2004) whereas the basal-like subtype is characterised by the absence of hormonal receptors and expression of basal cell markers, such as CK5, p63 or P-cadherin. In the present study, we have described a subset of carcinomas with down-regulation of CK19 expression, which lack ER, and associated with basal and/or myoepithelial cell differentiation. In fact, tumours negative for both CK19 and ER were always associated with the expression of at least one basal or myoepithelial cell marker. So, this subset might correspond to the so-called basal-like subtype, which has been found to be associated with a particularly poor clinical outcome in human patients (Sorlie *et al*., 2001; Abd El-Rehim *et al*., 2004; Nielsen *et al*., 2004; Rakha *et al*., 2006); however, additional studies are needed in order to confirm this hypothesis.

The loss of expression of luminal cytokeratins in conjunction with lack of ER expression, similar to that found in this study, has been described previously in human breast carcinomas (Abd El-Rehim *et al*., 2004; Willipinski-Stapelfeldt *et al*., 2005; Parikh *et al*., 2008). Choi *et al*. (2000) demonstrated that CK19 is under rapid and direct regulation by oestrogen in the MCF-7 breast cancer cell line, which suggest that cytoskeletal organisation might also be driven by hormonally-mediated stimuli in human breast cancer (Santini *et al*., 1996; Choi *et al*., 2000).

In the present study, we also performed a clinical follow-up analysis to assess CK19 prognostic significance and our results suggest that CK19 is associated with a more aggressive phenotype, since its down-regulation was significantly related with shorter OS and DFS. Previous studies on human breast cancer have made similar observations for luminal CK18 and 19 (Schaller *et al*., 1996; Woelfle *et al*., 2004; Parikh *et al*., 2008). However, a multivariate analysis including well-known prognostic variables showed that CK19 expression was not an independent prognostic factor in our series of canine mammary tumours, in contrast to human breast cancer (Parikh *et al*., 2008).

Considering that changes in the composition of the cytoskeleton of tumour cells may result in increased plasticity, which is required for epithelial tumour cells to become mobile and invasive (Thiery, 2002), luminal CK expression down-regulation might

among other factors lead to less differentiated tumour cells. Gene expression profile studies on breast cancer cell lines have found that expression of CK19 is consistently elevated in the less aggressive cell lines, whereas the highly aggressive cell lines expressed vimentin, a mesenchymal cell marker (Zajchowski *et al*., 2001). Similarly, Willipinski-Stapelfeldt *et al*. (2005) found that micrometastatic breast cancer cell lines displayed loss of luminal cytokeratins (CK8, CK18, and CK19) and showed an ectopic expression of vimentin, which is indicative of epithelial-mesenchymal transition.

Conclusions

Our findings demonstrate that CK19 down-regulation is associated with an aggressive tumour phenotype, identifying a group of dogs with higher risk of tumour progression. However, additional studies are warranted to confirm these findings and to investigate whether CK19 plays an active role, or whether the observed changes at the expression level merely reflect more upstream processes.

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Chapter VI

Identification of molecular phenotypes in canine mammary carcinomas with clinical implications: application of the human classification

Gama A, Alves A, Schmitt FC

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Identification of molecular phenotypes in canine mammary carcinomas with clinical implications: application of the human classification

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Abstract

Similarly to humans, canine mammary cancer represents a heterogeneous group in terms of morphology and biological behaviour. In the present study we evaluated a series of canine mammary carcinomas based on a new human classification, initially based on gene expression profiling analysis. Similarly to human breast cancer, by using an immunohistochemistry surrogate panel based on five molecular markers (estrogen receptor, HER2, cytokeratin 5, p63 and P-cadherin), we were able to classify canine mammary carcinomas into four different subtypes: luminal A (ER+/HER2-; 44.8%), luminal B (ER+/HER2+; 13.5%), basal (ER-/HER2- and a basal marker positive; 29.2%)and HER2 overexpressing tumours (ER-/HER2+; 8.3%). Luminal A-type tumours were characterized by lower grade and proliferation rate, whereas basal-type tumours were mostly high grade, high proliferative and positive for CK5, p63 and P-cadherin. In addition, as in humans, basal subtype was significantly associated with shorter disease-free and overall survival rates and we propose canine mammary carcinomas as a suitable natural model for the study of this particular subset of human carcinomas.

Keywords: canine, mammary carcinoma, immunohistochemistry, classification

Introduction

Mammary gland tumours are the most commonly occurring neoplasm in the female dog and represent a remarkably heterogeneous group in terms of morphology and biological behaviour [32, 43]. About half of canine mammary tumours are considered malignant and the identification of reliable prognostic factors is essential in order to estimate the individual risk of unfavourable clinical outcome [8, 29].

Several studies have recognized some reliable prognostic factors such as tumour size, histologic type, histologic grade and lymph node status [19, 30, 31]. Moreover, in recent literature we found an increasing number of investigations searching for suitable prognostic markers for canine mammary cancer [54], including proliferation markers [25], hormone receptors [23], p53 and Human Epidermal Growth Factor Receptor 2 (HER2) [21, 24] and adhesion molecules [14, 26], among others. The clinical experience is still limited, however, and reliable results of prospective studies are not always available.

Human and canine mammary cancer studies based on single molecular markers probably cannot accurately account for the heterogeneity of this disease [39]. Given the large number of cellular events involved in cell growth, differentiation, proliferation, invasion and metastases [4], the investigation of multiple molecular alterations in concert has been assuming great importance, due to the introduction of high-throughput technologies [39]. In fact, recent gene expression profiling studies on human breast tumours have identified distinct molecular subtypes of breast carcinomas which differ in their pathobiology and clinical outcomes [36, 47, 48]. Sorlie *et al*. [48] analyzed the expression profiles of 115 sporadic breast tumour samples and categorized them into five main groups: luminal A, luminal B, HER2-overexpressing, basal-like and normal breast tissue-like. Luminal A and B subtypes are based on the expression of estrogen receptor (ER), usually with luminal cytokeratin (CK) expression whereas the basal-like subtype is characterized by the absence of hormonal receptors and expression of basal cell markers [5, 33].

Given that gene expression profiling is impractical as a routine diagnostic tool, there are immunohistochemistry surrogate panels proposed that can potentially distinguish breast cancer subtypes [27, 33]. In the present study, we sought to identify phenotypical subtypes in canine mammary cancer with possible clinical implications. To accomplish this goal, we have characterized by immunohistochemical analysis a hundred and two

canine mammary carcinomas based on the immunohistochemical panel proposed by Matos *et al*. [27], which involved the evaluation of five molecular markers (ER, HER2, CK5, p63 and P-cadherin).

Material and methods

Tumour specimens

The present study is based on a series of a hundred and two cases of canine malignant mammary tumours (n=102) selected from the histopathological files of the University of Trás-os-Montes and Alto Douro, Vila Real and from the Institute of Biomedical Science at the University of Porto, Portugal. The material was fixed in 10% neutral formalin and embedded in paraffin wax. Sections (3 μm) were cut and stained with haematoxylin and eosin (HE) for histological examination, or used to perform immunohistochemistry.

Follow up data

Sixty nine cases (n=69) had available follow up data, with a median overall survival time of 15 months (range 5-74 months). Overall survival (OS) was defined as the period between surgery and animal natural death or euthanasia due to cancer. Disease-free survival (DFS) was defined as the period of time between surgery and recurrent or metastatic disease. During the follow up period, according to the referring surgeons, 35 animals died or euthanized due to metastatic disease and/or local recurrence.

Histological Examination

Tumours were diagnosed according to the WHO criteria for canine mammary neoplasms [31]. Clinicopathological variables included in the present study were: age, ovariohysterectomy status, contraceptive administration, tumour size, tumour location, tumour histological type and grade, presence of intra-tumoral necrosis, presence of vascular invasion and presence of lymph node metastasis.

Tumours were evaluated for grade in accordance with the Nottingham method for human breast tumours [11], based on the assessment of three morphological features: tubule formation, nuclear pleomorphism and mitotic counts. Each of these features was scored on a scale of 1 to 3 to indicate whether it was present in slight, moderate or

marked degree, giving a putative total of 3-9 points. Grade was allocated by an arbitrary division of the total points as follows: grade I (well differentiated), 3, 4 or 5 points; grade II (moderately differentiated), 6 or 7 points; and grade III (poorly differentiated), 8 or 9 points.

Immunohistochemistry

Tissue sections were incubated with primary monoclonal antibodies against ER, HER2, CK5, p63, P-cadherin and Ki-67. Table 1 summarizes the antibodies used and the staining procedures adopted for each antibody. Antigen retrieval was carried out by microwave treatment in a 10mM citrate buffer, pH 6.0, with the exception of P-cadherin, which was performed with an EDTA buffer, pH 8.0 (Lab Vision) in a boiling bath, during 20 minutes. For Ki-67, slides were previously incubated with 0.2 mg/mL trypsin (Merck) in phosphate buffered saline (PBS) for 10 min at 37ºC. After cooling (20 minutes at room temperature), the sections were immersed in 3% hydrogen peroxide (H_2O_2) and distillated water during 30 minutes to block endogenous peroxidase activity. All slides were then incubated with a blocking serum (Lab Vision, USA) for 10 min and then incubated with the specific antibody. After incubation, slides sections were incubated with biotinylated secondary antibody, followed by streptavidin-conjugated peroxidase (Lab Vision, USA), except for ER and HER2. For these antibodies, a polymeric labelling methodology was used as a detection system (Novolink Polymer Detection System, Novocastra, Newcastle, United Kingdom), following the manufacturer's instructions. Subsequently, the colour was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) and slides were counterstained with Gill's hematoxylin, dehydrated, and mounted for evaluation by light microscopy.

Adjacent normal mammary tissues were used as internal positive controls for CK5, p63, P-cadherin (basal and myoepithelial cells) and Ki-67. As positive controls, we also used canine uterus sections for ER and a human breast carcinoma with proved amplification (by FISH) and overexpression for HER2. Negative controls were carried out by replacing the primary antibody with PBS.

Table 1. Primary monoclonal antibodies and immunostaining protocols used.

Antibody	Origin	Clone	Dilution	Pretreatment	Incubation
ER	Novocastra, UK	NCL-LH2	1:40	Microwave	2h
HER2	Novocastra, UK	NCL-CB11	1:40	Microwave	Overnight
CK5	Neomarkers, USA	XM26	1:25	Microwave	Overnight
P63	Neomarkers, USA	4A4	1:150	Microwave	Overnight
PCAD	BD Transduction, USA	56	1:50	Water bath, 98ºC	Overnight
Ki-67	Dako, Denmark	Mib1	1:50	Trypsin + Microwave	Overnight

Evaluation of the immunohistochemical data

Nuclear ER immunoreactivity was considered positive when more than 10% of the neoplastic cells expressed this marker. To evaluate HER2 expression, Herceptest scoring system was applied (0=no membrane staining or <10% of cells stained; 1+=incomplete membrane staining in >10% of cells; 2+=>10% of cells with weak to moderate complete membrane staining; and 3+=strong and complete membrane staining in >10% of cells), with 2+ and 3+ cases considered positive. As for CK5 and p63, a semi-quantitative analysis was performed as follows: 0, <10% positive cells; 1, 10-50% positive cells and 2, >50% positive cells, with a cytoplasmic (CK5) or nuclear (p63) pattern of cellular distribution. Ki-67 and P-cadherin immunostainings were evaluated as previously described in canine tissues [14, 15]. CK5, p63 and P-cadherin were considered positive when more than 50% of the neoplastic cells expressed each marker.

Statistical Analysis

For statistical analysis, association between subtype tumour groups and continuous variables (mitotic and Ki-67 indices) was assessed with non parametric Kruskal-Wallis test. Associations between groups and categorical variables such as tumour size, histological type, histological grade and invasion were performed using the chi-square test. Survival curves were generated by the Kaplan-Meier method and the survival rates were compared using the log-rank test. All statistical analysis was performed using SPSS 11.5 statistical software. A P value <0.05 was considered statistically significant.

Results

Patients and tumour characteristics

The mean age of dogs at the time of surgical removal of tumours was 9.7 ± 2.5 years (range 4–16 years of age). The mean maximum tumour diameter was 4.21 ± 3.4cm (range 0.5-18cm), with tumours more frequently located in caudal mammary glands (n=36; 59%). In 10 (15.2%) out of the 66 female dogs with available clinical information, ovariohysterectomy (OHE) was performed prior to the removal of mammary tumours. Contraceptive administration was confirmed in 8 (13.8%) cases. Histological evaluation yielded 39 (42.4%) simple carcinoma, 41 (44.6%) complex carcinoma and 12 (13%) carcinosarcoma subtypes. According to the Nottingham method, tumours were classified as grade I (n=14, 15.2%), grade II (n=33, 35.9%) and grade III (n=45, 48.9%). Necrosis was present in 87 (94.6%) and vascular invasion in 51 (55.4%) cases. Lymph nodes were available in 49 cases, with confirmed metastasis in 26 cases (53.1%).

Immunohistochemistry profiles in canine tumours

The results of the immunohistochemical analysis performed for ER, HER2, CK5, p63 and P-cadherin are shown in Table 2 and Fig. 1. The immunohistochemical detection of ER was reliable in 96 cases: the remaining tumours have lost ER antigenicity (adjacent mammary gland was negative) and were excluded. Immunohistochemical evaluation of HER2 and P-cadherin was available in 100 and 96 cases, respectively.

ER and p63 positive cases showed the characteristic nuclear staining, whereas CK5 positive ones showed a cytoplasmic pattern of expression. HER2 positive tumours showed a membranous staining and P-cadherin positive tumours showed a cytoplasmic and/or membranous immunostaining. We observed that 58.3% of canine mammary carcinomas in our series were ER positive, whereas 21% were HER2 positive (2+ and 3+). A positive basal cell marker expression was present in 32.4% tumours for both CK5 and p63 and in 42.8% tumours for P-cadherin.

According to Nielsen *et al*. [33], we classified each tumour based on its ER and HER2 expression. A total of 96 cases were immunohistochemically interpretable to allow sample characterization into one of five categories (Table 3). If a tumour was ER

positive, it was classified as luminal; moreover, we distinguish luminal A and B on the basis of HER2 overexpression. If a tumour was ER positive and HER2 negative (0 or 1+), it would be classified as luminal A (ER+/HER2-); however, if it was ER and HER2 positive, it would be classified as luminal B (ER+/HER2+). If a tumour was ER negative and HER2 positive (ER-/HER2+), it would be classified as HER2-overexpressing, and if it was both ER- and HER2- negative but positive for at least one basal marker (CK5 and/or p63 and/or P-cadherin), it would be classified as basal (ER-/HER2-). If a tumour did not show expression for any of these markers, it would be classified as negative (null phenotype) and would not be considered in the remaining analyses.

Using this definition, we observed that luminal A and B subtypes comprised 44.8 and 13.5% of all tumours, respectively; basal subtype comprised 29.2%; HER2 overexpressing subtype represented 8.3% and negative/null phenotype accounted for 4.2% in this tumour series (Table 3).

Table 2. Imunohistochemical results in the present study.

Molecular marker	Positive staining n (%)	Negative staining n (%)
ER[a]	56 (58.3)	40 (41.7)
HER2[a]	21 (21)	79 (79)
CK5	33 (32.4)	69 (67.6)
P63	33 (32.4)	69 (67.6)
PCAD[a]	42 (42.8)	54 (56.3)

[a] Immunohistochemical evaluation of ER and P-cadherin was available in 96 cases and HER2 was available in 100 cases.

Table 3. Frequencies of immunohistochemically defined subtypes of canine mammary carcinomas (n=96).

Subtype	ER	HER2	P-CD and/or p63 and/or CK5	Frequency [n (%)]
Luminal A	Positive	Negative	Positive/negative	43 (44.8%)
Luminal B	Positive	Positive	Positive/negative	13 (13.5%)
Basal	Negative	Negative	Positive	28 (29.2%)
HER2-overexpressing	Negative	Positive	Positive/negative	8 (8.3%)
Negative/null phenotype	Negative	Negative	Negative	4 (4.2%)

Statistically strong significant differences between the four groups were observed in this study, when related with some relevant clinicopathological parameters (Table 4). Basal and HER2 overexpressing subtypes were associated with simple or carcinosarcoma histological types, whereas complex carcinomas were mostly of luminal A subtype ($P<0.0001$). In addition, basal subtype tumours presented higher histological grade, representing 55.6% of grade III tumours ($P<0.0001$) and were also significantly associated with the presence of vascular invasion ($P<0.0001$).

Basal marker expression clearly differed across distinct molecular subtypes (Table 5). Basal and HER2-overexpressing tumours demonstrated a higher frequency of the basal cell markers p63 and P-cadherin ($P<0.0001$ and $P=0.001$) and CK5 positive tumours were frequently basal subtype tumours ($P=0.001$). In contrast, luminal pattern was associated with a lower expression of basal markers. In fact, when analysing basal marker expression simultaneously, we found that the majority of luminal tumours were simultaneously negative to CK5, p63 and P-cadherin. All HER2-overexpressing tumours expressed at least one basal marker and the basal subtype tumours showed frequently the expression of two or all basal markers ($P<0.0001$).

With regard to proliferation indices, luminal A tumours showed lower median mitotic and Ki-67 labelling indices ($P=0.001$ and $P<0.0001$, respectively), whereas all other groups were characterized by higher proliferation rates, with basal subtype showing the highest Ki-67 index.

Follow up data revealed that basal subtype was significantly associated with lower overall ($P=0.002$, Fig. 2A) and disease-free ($P=0.01$, Fig.2B) survival rates, whereas the other groups showed higher survival rates, including the HER2-overexpressing group.

Discussion

Recently, gene expression profiling has redefined breast cancer taxonomy and identified five distinct subtypes of carcinomas: luminal A, luminal B, normal breast-like, HER2 overexpressing and basal-like [36, 47, 48, 53]. These molecular subtypes not only reflect the heterogeneity of breast carcinomas and the possible different cell lineage pathways in breast carcinogenesis, but also demonstrate the difference in clinical outcome, with basal-like subtype associated with a more aggressive behaviour [1, 47, 48, 52, 53].

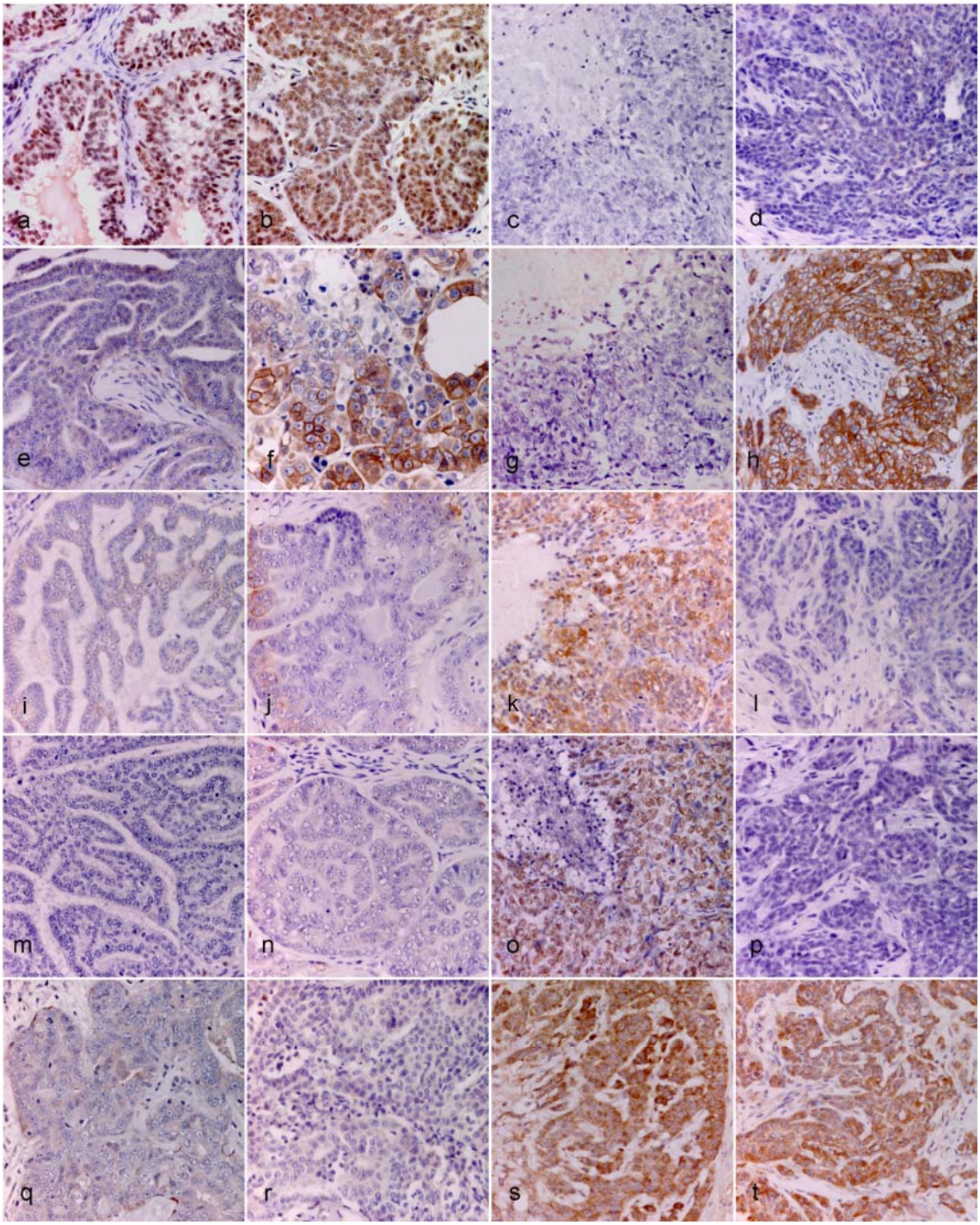

Fig. 1 Immunohistochemical expression of the different proteins studied by IHC in canine mammary carcinomas. **a-d** ER staining; **e-h** HER2 staining; **i-l** CK5 staining; **m-p** p63 staining; **q-t** P-cadherin staining. Each column represents a distinct molecular subtype. From left to right, each column represents luminal A, luminal B, basal and HER2 overexpressing subtypes. [original magnification x400]

Table 4. Association between tumour subtypes and clinicopathological characteristics.

	Luminal A [n (%)]	Luminal B [n (%)]	Basal [n (%)]	HER2 overexpressing [n (%)]	*P*
Age					
≤ 9 years old	18 (43.9%)	6 (14.6%)	13 (31.7%)	4 (9.8%)	0.90
> 9 years old	24 (51.1%)	6 (12.8%)	14 (29.8%)	3 (6.4%)	
Tumour size					
<3 cm	17 (53.1%)	6 (18.8%)	8 (25%)	1 (3.1%)	0.37
3-5 cm	14 (46.7%)	4 (13.3%)	8 (26.7%)	4 (13.3%)	
>5 cm	9 (39.1%)	1 (4.3%)	10 (43.5%)	3 (13%)	
Tumour location					
Cranial glands	2 (50%)	0 (0%)	1 (25%)	1 (25%)	0.09
Medial gland	6 (60%)	2 (20%)	1 (10%)	1 (10%)	
Caudal glands	12 (33.3%)	4 (11.1%)	10 (50%)	2 (5.6%)	
Multiple	8 (72.7%)	2 (18.2%)	0 (0%)	1 (20%)	
Ovariohysterectomy					
No	18 (39.1%)	7 (15.2%)	17 (37%)	4 (8.7%)	0.057
Yes, prior to tumour development	9 (90%)	0 (0%)	0 (0%)	1 (10%)	
Yes, performed with mastectomy	6 (60%)	0 (0%)	4 (40%)	0 (0%)	
Contraception					
No	22 (44%)	6 (12%)	17 (34%)	5 (10%)	0.36
Yes	6 (75%)	0 (0%)	2 (25%)	0 (0%)	
Histological type					
Simple carcinoma	9 (23.1%)	8 (20.5%)	17 (43.6%)	5 (12.8%)	**<0.0001**
Complex carcinoma	32 (78%)	5 (12.2%)	3 (7.3%)	1 (2.4%)	
Carcinosarcoma	2 (16.7%)	0 (0%)	8 (66.7%)	2 (16.7%)	
Histological grade					
Grade I	14 (100%)	0 (0%)	0 (0%)	0 (0%)	**<0.0001**
Grade II	23 (69.7%)	5 (15.2%)	3 (9.1%)	2 (6.1%)	
Grade III	6 (13.3%)	8 (17.8%)	25 (55.6%)	6 (13.3%)	
Necrosis					
Absent	4 (80%)	1 (20%)	0 (0%)	0 (0%)	0.29
Present	39 (44.8%)	12 (13.8%)	28 (32.3%)	8 (9.2%)	
Lymphovascular Invasion					
Absent	29 (70.7%)	6 (14.6%)	4 (9.8%)	2 (4.9%)	**<0.0001**
Present	14 (27.5%)	7 (13.7%)	24 (47.1%)	6 (11.8%)	
Lymph node metastasis[a]					
Absent	13 (56.5%)	6 (26.1%)	3 (13%)	1 (4.3%)	0.1
Present	8 (30.8%)	5 (19.2%)	11 (42.3%)	2 (7.7%)	

[a]Lymph nodes were available in 49 cases.

Table 5. Association between different subtypes *versus* basal marker expression and proliferation indices.

	Luminal A [n (%)]	Luminal B [n (%)]	Basal [n (%)]	HER2 overexpressing [n (%)]	*P*
CK5					
Negative	36 (60%)	8 (13.3%)	11 (18.3%)	5 (8.3%)	**0.001**
Positive	7 (21.9%)	5 (15.6%)	17 (53.1%)	3 (9.4%)	
P63					
Negative	32 (53.3%)	13 (21.7%)	11 (18.3%)	4 (6.7%)	**<0.0001**
Positive	11 (34.4%)	0 (0%)	17 (53.1%)	4 (12.5%)	
P-cadherin					
Negative	26 (56.5%)	10 (21.7%)	9 (19.6%)	1 (2.2%)	**0.001**
Positive	13 (31.7%)	3 (7.3%)	18 (43.9%)	7 (17.1%)	
Basal markers					
All negative	21 (77.8%)	6 (22.2%)	0 (0%)	0 (0%)	**<0.0001**
One positive	11(39.3%)	6 (21.4%)	8 (28.6%)	3 (10.7%)	
Two positive	5 (20%)	1 (4%)	15 (60%)	4 (16%)	
All positive	2 (25%)	0 (0%)	5 (62.5%)	1 (12.5%)	
Median Mitotic index[a] (Min-Max)	0.44 (0-1.59)	1.0 (0.1-2.99)	0.94 (0.1-2.09)	0.7 (0.3-1.9)	**0.001**
Median Ki-67 index[a] (Min-Max)	17.89 (5.39-56.36)	26.7 (15-44.8)	28.14 (12.10-49.2)	26.4 (22.5-35.86)	**<0.0001**

[a] Proliferative indices were available in 86 cases.

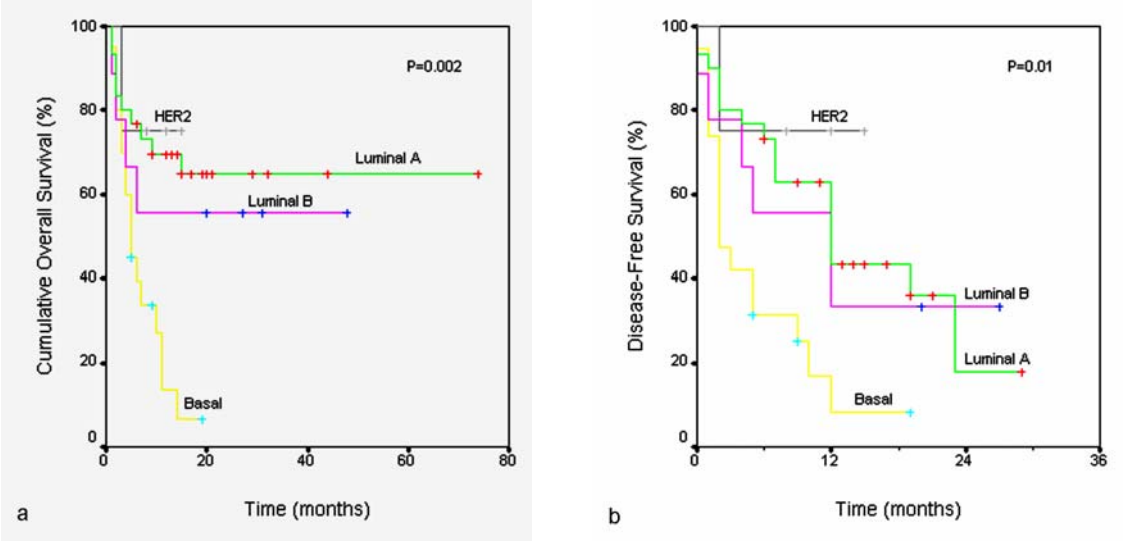

Fig. 2 Kaplan-Meier overall survival (A) and disease-free survival (B) curves of the different subtype groups.

Although gene expression profiling is still considered the "gold standard" for the identification of breast carcinoma subtypes, this technology requires highly sophisticated technical equipment and is not readily available for clinical application or for retrospective studies using formalin fixed, paraffin-embedded samples [39]. For this reason, immunohistochemistry has been used in several studies and the evaluation of a limited panel of immunohistochemical cell markers have shown that breast carcinomas can be subdivided into subgroups remarkably similar to the ones defined by gene expression profiling [1, 3, 22, 27, 33, 38, 52].

In the present study we found in a series of canine mammary tumours, similar findings observed in human breast cancer. We have also identified distinct phenotypical subtypes in a series of canine mammary carcinomas, by using an immunohistochemical panel which included five molecular markers (ER, HER2, CK5, p63 and P-cadherin). Based on ER/HER2 molecular classification, we defined four main subgroups: luminal A (ER+/HER2–, 44.8%), luminal B (ER+/HER2+, 13.5%), basal-like (ER–/HER2–, 29.2%) and HER2 overexpressing (ER–/HER2+, 8.3%). In contrast, Sarli *et al*. [44] have only identified luminal A and B subtypes when studying a series of 39 canine mammary carcinomas. Although using a similar terminology, they used a distinct panel of molecular markers and the adopted classification was not identical, with luminal subtype defined as CK19 positive tumours, regardless of hormonal status (luminal A, HER2– and luminal B, HER2+), and HER2 overexpressing and basal-like subtypes defined as CK19 negative tumours, HER2+ and HER2–, respectively.

In the current study, we found statistically strong significant differences between the four groups, with ER positive luminal A tumours more frequently associated with complex tumour type, low histological grade, less invasive and low proliferative tumours, whereas basal-like and HER2 overexpressing subtypes were associated with simple and carcinosarcoma tumour types, high histological grade, lymphovascular invasion and high proliferation, features that are in accordance to the ones described in recent human literature for basal-like cancers [20, 22, 27, 40].

CK5, p63 and P-cadherin are proteins that are expressed early in epithelial differentiation and may contribute to a committed stem cell and/or progenitor phenotype [6, 7, 9, 35]. In this study, we demonstrate that these markers are upregulated in the basal subtype, similarly to Matos *et al*. previous results [27]. In fact, the basal subtype rarely expressed just one basal marker but frequently expressed them simultaneously,

which suggests a more undifferentiated profile. HER2-overexpressing subtype was also characterized by an up-regulation of basal markers, confirming some human breast studies which suggested that HER2-overexpressing tumours should be included in a bona fide basal-like subclass [5, 27]. In contrast, the majority of luminal tumours in our series were simultaneously negative for basal cell markers, with some cases showing basal marker expression, which was also described by some authors, who reported tumours co-expressing basal CK and hormone receptors or HER2 [40, 50].

Similarly to human breast cancers, in this study we further demonstrate the molecular heterogeneity of canine mammary cancer. A "hierarchy or stem cell" model of breast cancer oncogenesis has been proposed to elucidate the observed functional heterogeneity of tumours. In this model, transformation occurs in a stem cell, or in a progenitor "highly proliferating" cell, and expansion proceeds until various maturation stages, depending on the genomic alterations. Specific genetic alterations would lead to distinct cellular transcriptomic programmes, including the change of hormonal receptors and CK expression pattern, characterising distinct subgroups of breast carcinomas (5, 8, 39].

Survival analysis revealed that distinct subtypes were associated with different clinical outcomes, with basal subtype associated with lower survival rates, similarly to human breast cancer studies [36, 47, 48]. These results also corroborate a previous study in canine mammary cancer performed by Griffey *et al*. [16] which firstly described basal carcinomas as having poor prognostic features. Despite many different studies associating basal-like tumours with a more aggressive clinical history and shorter survival [3, 33, 37, 47, 48, 49, 52], others did not find such a prognostic significance [12, 18]. These variations are probably related to differences between studies in patient cohorts, analytic methods and, most importantly, the immunohistochemical definitions of basal-like breast cancer [39]. Recently, Tang *et al*. [51] comparing several classifications with similar terminology but different definitions (such as ER/HER and triple negative classification) concluded that these classifications are related but not interchangeable.

In contrast to basal subgroup, luminal and HER2 overexpressing subtypes showed increased survival rates. The fact that luminal tumours were associated with a better prognosis is not surprising, since ER positive human breast carcinomas are usually associated with a more favourable clinical outcome. In veterinary pathology, however,

the prognostic value of ER in canine mammary cancer is still a matter of debate. Previous studies using biochemical [45] and immunohistochemical [34] methodologies have demonstrated the prognostic value of ER, but others have failed this confirmation [23, 28]. The observed discrepancies between different studies are probably related with sample selection, differences in antibodies, staining procedure and evaluation or sensitivity of the detection system. In our series, luminal tumours were mostly of complex type, which comes in accordance to previous canine studies reporting complex carcinomas as being more likely ER positive [15, 23, 28]. Given that this tumour type is generally associated with a better clinical outcome, its high proportion in luminal subtype groups is probably in part responsible for their favourable prognosis.

Despite HER2 recognition as a prognostic factor in human breast cancer [41, 46], the significance of HER2 overexpression in dogs with mammary carcinoma is still unclear. Some studies have shown that either HER2 amplification or protein overexpression are present in canine mammary carcinomas [2, 42], while others found no gene amplification [24]. Similarly to previous studies [10, 24], HER-2 overexpressing tumours were found usually associated with established indicators of poor prognosis such as large tumour size, high histologic grade, invasion, simple histologic type and high proliferative indices. However, Kaplan-Meier analysis revealed that this subtype was related with a more favourable clinical outcome, findings that are in contrast with human studies, which describe similar survival rates for HER2 overexpressing and basal-like subtypes [36, 47, 48], and are probably related to the small number of cases that comprise the HER2 overexpressing subtype. However, a recent study performed by Hsu *et al*. [17] revealed that HER2 overexpression in canine malignant mammary tumours is associated with higher survival rates. Additional large-scale studies are warranted to further explore the value of HER2 in canine mammary carcinomas.

In conclusion, as in humans, our study defined distinct molecular phenotypes in canine mammary carcinomas based on immunohistochemical analysis. Moreover, we have identified a basal-like subtype representing almost 30% of our series, which was associated with a more aggressive clinical behaviour. We believe that canine mammary carcinomas would be suitable natural models for the study of this particular subset of carcinomas. However, more studies are needed regarding the prognostic value of these immunohistochemically determined subtypes in canine mammary cancer.

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Chapter VII

General Discussion and Concluding Remarks

General Discussion and Concluding Remarks

Until recently, the main role of the veterinary pathologist laid on the establishment of a basic diagnosis. In the context of canine mammary tumours, apart from the examination of regional lymph nodes for the presence or absence of metastasis it was unusual for any other prognostic information to be supplied, or indeed, requested. Nowadays, veterinary oncologists want to know not only the standard histological features of mammary tumours, such its type and grade, but also relevant information concerning the prognosis of an individual animal.

Canine mammary cancer represents a very important disease and there has been a persistent drive in order to identify reliable prognostic factors. The prognostic value of clinicopathological parameters in canine mammary tumours is still a matter of debate and the controversial results obtained in a rather small number of prognostic studies also prevent the routine use of molecular markers. The conducted studies included in this thesis aimed to study the prognostic impact of several clinicopathological and molecular characteristics of canine mammary gland tumours.

With regard to clinicopathological parameters, several variables were found of prognostic value on univariate analysis, including tumour size, ulceration, histological type, tumour growth pattern, histological grade, stromal/vascular invasion and lymph node status. Thus, large sized tumours, skin ulceration, simple and carcinosarcoma histological type, infiltrative tumour growth and the presence of stromal/vascular invasion and node metastasis were significantly associated with reduced survival rates. In addition, high proliferation indices (both mitotic and Ki-67 indices) were also associated with poorer survival times.

The histological heterogeneity observed in canine mammary neoplasms presents considerable difficulties in the design of a classification system that will assure reproducibility of a prognostically meaningful categorization (Gilbertson *et al*., 1983). In our study, the categorization of mammary malignant tumours in simple carcinoma, complex carcinoma and carcinosarcoma types has been associated with distinct biological behaviours. As previously observed by de las Mulas and Peña (2004), we have also found some difficulties in the application of the new WHO tumour classification, mostly in the definition of carcinoma in benign tumour. In the present study, these tumours were included in the complex carcinoma group, given that they

were characterized by an extensive complex phenotype, although some minor areas of metaplastic changes occurred in a few cases. In addition, these tumours showed very similar clinical behaviour with other complex carcinomas. As for simple carcinoma group, it harboured several distinct morphological entities and it remains our goal to gather a large number of tumour cases in order to perform a prospective analysis considering separate histological types. Even so, simple carcinomas and carcinosarcomas were strongly associated with shorter survival rates, when compared to complex carcinomas. Ongoing work includes the establishment and characterization of canine mammary cell lines of epithelial and myoepithelial differentiation, in order to unravel myoepithelial cell putative role as a natural invasion tumour suppressor in canine mammary tumours.

Post-surgical prognosis has been the subject of several prospective studies, but only a few multivariate analyses have been carried out to determine which clinicopathological parameters have independent prognostic value (Hellmén *et al*., 1993; Peña *et al*., 1998; Nieto *et al*., 2000; Chang *et al*., 2005; Itoh *et al*., 2005; de Matos *et al*., 2006). On multivariate Cox-regression analysis, only lymph node status represented an independent prognostic factor, which stresses the critical importance of the standard availability of regional lymph nodes. Based on our findings, we confirm that veterinary pathologists are in an ideal position to supply clinical colleagues with a substantial amount of useful prognostic information, just from the routine examination of canine mammary tumours.

When searching for suitable prognostic markers, it is important to focus on cell adhesion properties, which might be related to the cells' ability to detach from neighbouring cells leading to the first steps of invasion and metastasis (Zaidan Dagli, 2008). Inspired by this idea, we have investigated the immunohistochemical expression of the cell adhesion molecules E-cadherin, P-cadherin and β-catenin in a series of canine mammary malignant tumours, and their relationship with clinicopathological parameters, proliferation and survival. Our study revealed that a reduced E-cadherin and β-catenin expression was significantly associated with several aggressive clinicopathological features, such as high histological grade and invasion, as shown in previous canine mammary studies (Brunetti *et al*., 2005; Matos *et al*., 2006; de Matos *et al*., 2007). In addition, abnormal E-cadherin and β-catenin expression was significantly associated with poorer survival times, in contrast to Brunetti *et al*. findings (Brunetti *et*

al., 2005). From our results, we confirmed that an altered expression of the cadherin-catenin complex is associated with cell invasion and might play a central role in canine mammary tumour progression. However, to further validate E-cadherin and β-catenin molecules as prognostic markers, additional studies are warranted, including a larger series of tumours and a longer follow-up period.

Similarly to canine mammary gland tumour findings, E-cadherin expression studies have revealed some contradictory results in human breast cancer, where some authors failed to reveal a prognostic value. In fact, some breast cancers with aggressive characteristics present high levels of E-cadherin and many metastases are E-cadherin-positive (Shiozaki *et al.,* 1996; Howard *et al*., 2005). Thus, the expression of E-cadherin is likely to be dynamic; it is possible that temporary or localized downregulation of E-cadherin promotes detachment of cells from the primary tumour and invasion into the local environment and that posterior re-expression of E-cadherin in a new environment might foster their survival as they are carried to a distant site (Knudsen and Wheelock, 2005).

Another consideration is that E-cadherin, even if it is expressed in mammary cancers, is not fully functional unless it forms a complex with catenins and anchors to the cytoskeleton. In this study we have investigated both E-cadherin and β-catenin, considering a separate and a combined expression of these molecules, and we have confirmed that idea, given that the loss of at least one of these proteins was associated with an aggressive tumour phenotype. Additional studies are required in order to shed some light on E-cadherin regulation and signalling in canine mammary tumours. Recent investigations point out to regulatory signals between oestrogen and E-cadherin, with loss of oestrogen resulting in its subsequent repression (Fujita *et al*., 2003; Park *et al*., 2008). In fact, it was recently described an association between E-cadherin absence/reduction and a basal-like phenotype in human breast carcinomas (Mahler-Araujo *et al*., 2008).

As for P-cadherin, to the best of our knowledge, no study had previously studied its prognostic value in canine mammary tissues. P-cadherin expression was only significantly associated with an invasive tumour phenotype, with no association with survival. Our results are discordant with the majority of available studies in human breast cancer, which found P-cadherin significantly associated with several aggressive characteristics, such as high histological grade and proliferation, as well as with a poor

prognosis (Peralta Soler *et al*., 1999; Gamallo *et al*., 2001; Arnes *et al*., 2005). Yet, in this first study we have analysed a relatively small number of cases. Later on, we have expanded our series and we have found P-cadherin expression significantly associated with high histological grade and with a poor prognosis, being strongly associated with a basal-like phenotype. However, although P-cadherin positive carcinomas indeed appear to have a myoepithelial/basal-like transcriptomic programme, this explanation is unlikely to account for the high percentage of P-cadherin expressing tumours, as suggested for human breast. It is easier to accept that some molecular mechanisms would lead to activation of P-cadherin expression (Paredes *et al*., 2007). In fact, a significant correlation was recently described between P-cadherin expression and hypomethylation of a specific region of the *CDH3* promoter, suggesting an important regulatory role for cytosine methylation in the aberrant expression of P-cadherin in breast cancer (Paredes *et al*., 2005). On the other hand, the lack of ER signalling was found responsible for the increase in P-cadherin, categorizing *CDH3* as an oestrogen-repressed gene and pointing to E2 as a key regulator of this cadherin (Paredes *et al*., 2004). As already discussed, the role of P-cadherin in breast cancer remains incompletely understood. Whether it represents a useful prognostic marker or plays a causal role is open to question, both in canine and in the human species.

The availability of molecular targeted therapies that interfere with specific targets having critical roles in tumour growth and progression is promising for cancer treatment, and the recent availability and US Food and Drug Administration approval of specific EGFR tyrosine kinase inhibitors has increased the interest on this growth factor receptor in human breast cancer studies (Baselga and Arteaga, 2005; Jorgensen *et al*., 2007; Widakowich *et al*., 2007). In the present thesis, we report for the first time an immunohistochemical study of EGFR expression in benign and malignant canine mammary gland tumours, confirming previous biochemical findings at the cellular level. Our results have found EGFR expression significantly associated with a malignant tumour phenotype. However, EGFR was not significantly associated with clinicopathological variables, other than animal age and tumour size. In addition, although dogs affected by EGFR-overexpressing malignant tumours showed poorer survival rates compared to dogs harbouring EGFR negative tumours, the differences observed failed to reach statistically significant levels. Given the tendency of positive EGFR cases towards poor prognosis, the possibility exists that a number of dogs might

benefit from EGFR-targeted therapy, as in human breast and lung cancer patients (Lambros *et al*., 2007; Faratian and Bartlett, 2008). However, to test this hypothesis and to find out if this receptor has prognostic value in canine mammary cancer, additional studies are warranted with a larger series of tumours and follow-up analysis.

Previous studies concerning EGFR expression in canine mammary samples were performed by using biochemical assays (Nerurkar *et al*., 1987; Donnay *et al*., 1993; Rutteman *et al*., 1990, 1994; Donnay *et al*., 1996), with no differences observed between benign and malignant tumours or with clinicopathological parameters. The distinct approach used between our and previous canine studies makes difficult a direct comparison. Similarly, in human literature, conflicting results are also found, and EGFR is not a consensual prognostic marker. The lack of standardized assessment method and interpretation criteria for EGFR expression may contribute to these apparent contradictory findings (Klijn *et al*., 1992; Bhargava *et al*., 2005; Park *et al*., 2007). At a practical level, there is no universal method for evaluating EGFR expression in human breast tumours and it is of particular interest to disclose if EGFR expression levels can really predict the response to therapy, keeping in mind that EGFR signalling network is comprised of a complex series of interconnecting pathways and each component is likely to affect the level of EGFR signalling output (Ciardiello and Tortora, 2003; Milanezi *et al*., 2008).

In the near future, we intend to study the underlying mechanisms of EGFR protein overexpression, such as *EGFR* gene amplification, in canine mammary tumours. It is possible that, similarly to the findings in human breast carcinomas, gene amplification does not represent the main mechanism, but this remains unknown in the canine species. On the other hand, several authors have suggested other regulatory mechanisms, namely at transcriptional level (Berquin *et al*., 2001, 2005; Kersting *et al*., 2004; Milanezi *et al*., 2008), which can be related with specific cellular transcriptomic programmes, such as myoepithelial/"basal-like" differentiation (Reis-Filho *et al*., 2005). Given that EGFR is consistently expressed in normal canine myoepithelial cells, further studies are required to elucidate if EGFR is associated with a basal-like phenotype in canine tumours, as it has been shown in human breast cancer.

Additionally, we have studied the expression of several cell differentiation markers in a series of canine mammary malignant tumours and we have identified a subset of canine mammary carcinomas with lack or reduction of CK19 epithelial expression, a luminal

epithelial cell marker, strongly associated with the expression of basal and/or myoepithelial cell markers. A reduced or absent CK19 expression was also significantly associated with several clinicopathological variables, such as invasion and high histological grade, as well as with high proliferative index.

To our knowledge, this is the first study dealing with CK expression in canine mammary carcinomas in which survival analyses have been performed. These analyses revealed a less favourable disease course for tumours with a basal phenotype than for those with a luminal phenotype, identified by CK19 expression. However, Cox regression multivariate analysis has not revealed CK19 as an independent prognostic variable in canine mammary malignant tumours. It remains to be elucidated if CK19 is merely a reflection of cell differentiation or if it plays an active role during tumour progression. Future studies involving larger number of cases will be needed before such questions can be satisfactorily answered.

In human breast cancer studies, a high level of luminal CK (CK8, CK18 or CK19) immunostaining has been also correlated with a more favourable prognosis (Takei *et al*., 1995; Schaller *et al*., 1996; Woelfle *et al*., 2004; Parikh *et al*., 2008). However, contradictory findings were described in distinct tumours, since it was recently suggested that the expression of CK19 in pancreatic endocrine tumours may be correlated with a poor prognosis (La Rosa *et al*., 2007).

The biological significance of the expression of basal CK in poorly differentiated canine mammary carcinomas remains an enigma. One hypothesis is that its expression might indicate derivation from, or toward, myoepithelial cells. In fact, the expression of the smooth muscle actin and calponin has been observed in several carcinoma cases, as previously described in human cancer studies (Santini *et al*., 1996; Tsuda *et al*., 2000).

Both canine and human mammary cancer has been recognized as a heterogeneous disease in terms of morphology and biological behaviour. Recent studies based on gene expression profiling have reflected human breast cancer heterogeneity at the molecular level and lead the way into modern breast cancer taxonomy (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003). The introduction of high-throughput microarray technology allowed the analysis of the expression levels of thousands of genes and the distinction of breast cancer subclasses with diverse biological behaviours (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003; Sotiriou *et al*., 2003). Several immunohistochemical studies have reinforced this novel breast cancer taxonomy at the protein level, by using a surrogate

panel of immunohistochemical markers (Nielsen *et al*., 2004; Matos *et al*., 2005; Rakha *et al*., 2008). In this study, we have applied this novel classification on canine mammary carcinomas and we have identified similar molecular subtypes to the ones found in human breast cancer, by using a surrogate panel of immunohistochemical cell markers, which included ER, HER-2, CK5, P63 and P-cadherin. Four main subtypes were identified: luminal A, luminal B, HER-2 overexpressing and basal-like, with basal-like subtype significantly associated with poor prognosis.

Currently, in the human setting, hormonal and HER-2 status are routinely used to predict prognosis and to determine a patient's specific treatment (Payne *et al*., 2008). Looking at the situation in dogs, we are far behind from humans; no consensus was reached on the prognostic value of ER or HER-2 status and surgery still remains the treatment of choice for most dogs with mammary gland tumours (Rutteman *et al*., 2001).

Our study confirmed the reliability of monoclonal antibodies directed against ER and HER-2 proteins in routinely processed formalin-fixed canine mammary tissues, by using a highly sensitive polymeric detection system. We have demonstrated that ER expression (luminal subtypes) is significantly associated with better survival rates, confirming some previous results (Nieto *et al.,* 2000). Future prospective studies are required to validate ER as a prognostic and predictive marker in canine mammary tumours. In fact, the possibility exists that ER may still represent a rationale therapeutic target in canine mammary cancer (Soremno, 2003).

HER-2 status also needs further consideration in subsequent studies. Similarly to EGFR, HER-2 also represents an appealing target molecule for cancer therapy. As already described elsewhere in this thesis, the development of trastuzumab, a humanized monoclonal antibody against HER-2 extracellular domain, settled HER-2 importance as a therapeutic target in human breast cancer (Milanezi *et al*., 2008), and this fact opens up the possibility of using HER-2 directed therapies in canine metastatic cancer. However, in the present investigation, HER-2 overexpressing tumours were associated with increased survival rates, which is in accordance to the recent results of Hsu and co-workers (2007) in canine tumours but largely contradicts the available human literature (Slamon *et al*., 1987). Given that our series comprised a small number of HER-2 overexpressing tumours, additional prospective studies are in order to further explore HER-2 prognostic value.

The identification of a subset of carcinomas with a basal-like phenotype confirms, at some point, a previous study performed by Griffey and co-workers (1993), which used this terminology for the first time in canine mammary carcinomas. In the present series, basal-like phenotype was significantly associated with a high histological grade and proliferation, as well as with shorter disease-free and overall survival rates. It is not known to what extent basal-like phenotype represents a signature derived from the cell of origin of these cancers, or more is the result of differentiation from a precursor that is common to all breast cancers and therefore does not reflect histogenesis. In fact, two hypothetical models of mammary oncogenesis showing the potential origin of breast cancer cell have been proposed. The "stochastic" model suggests that clonal tumour expansion originates from any cell, whatever its stage of differentiation, after it has been randomly hit by enough genomic alterations to trigger transformation; the tumor cell acquires a self-renewing capacity but preserves characteristics of its origin. The "hierarchy" or "stem cell" model suggests that transformation occurs in a stem cell, or in a progenitor cell, and expansion proceeds concomitantly to usual maturation until various stages, depending on the identity of genomic alterations (Birnbaum *et al*., 2004; Rakha *et al*., 2008).

In human studies, it has been shown that the basal-like subtype is a rather heterogeneous tumour group. Although basal-like tumours are mainly high grade invasive ductal carcinomas, showing morphological characteristic features, such as large central necrotic areas, pushing margins of invasion, high-grade nuclear features and high mitotic index (Livasy *et al*., 2006; Rakha *et al*., 2006), other histological types display a basal-like phenotype, namely medullary carcinomas, metaplastic carcinomas and myoepithelial type carcinomas (Rakha *et al*., 2008). Our next step will be a thorough characterization of this basal-like subtype in canine mammary cancer, performing a large scale study of canine mammary tumours, which will also verify the robustness and independent significance of the present findings.

To further substantiate this classification in canine mammary cancer, the analysis of gene expression levels by high-throughput microarray technology will certainly be the ultimate proof. However, this methodology is expensive, requires access to large numbers of fresh frozen tumour samples and is impractical as a routine diagnostic tool (Rakha *et al*., 2008). Very recently, a cDNA microarray study was conducted on three canine mammary tumour cell lines, revealing distinct gene expression profiles

pertaining to their phenotype. However, these authors were not able to directly compare canine cell data with human gene sets because of a lack of representation of these genes on the canine microarray. Even so, pathway analysis identified a striking similarity in the pathway profiles of canine mammary tumour, human breast cancer cell line and breast carcinoma intrinsic gene sets (Rao *et al*., 2008).

A major challenge nowadays is to identify therapeutic targets for the basal-like subtype of human breast cancer, which is not responsive to endocrine therapy or HER-2-directed therapy. Despite the extensive research on this tumour phenotype, the specific genes that drive its aggressive behaviour are poorly understood. A number of attractive gene products have been identified by gene expression profiling in the basal-like cluster, some of them implicated in cellular proliferation, suppression of apoptosis or cell invasion (Perou *et al*., 2000; Sorlie *et al*., 2001). These gene products include EGFR, αB-crystallin, TGFβ2, MMP14, cyclin E1 and c-KIT, and although quite diverse, several activate similar signalling pathways such as MAPK–ERK and PI3-kinase–AKT pathways, which may play a central role in the pathogenesis of basal-like carcinomas (Vogelstein and Kinzler, 2004; Yehiely *et al*., 2006). This specific basal gene profile provides several potential targets for therapy, namely EGFR and its downstream signalling pathways (Siziopikou and Cobleigh, 2007). It is our objective to study EGFR expression and amplification in the basal-like subset of canine carcinomas, in order to investigate this molecule as a potential therapeutic target. Although we consider the present findings as preliminary results, canine mammary carcinomas might represent a suitable natural model for the study of human breast carcinomas, in particular to the basal-like subset, given the putative high percentage of basal carcinomas identified in the canine species.

The main goal of prognostic studies is to identify reliable prognostic factors, which might be used in the routine setting, preferably with therapeutic potential. We hope that our present study on canine mammary tumours would contribute in some way in the understanding of this complex disease. From our results, we stress the following major conclusions:

1. With regard to clinicopathological parameters, univariate analysis revealed that tumour size, ulceration, histological type, tumour growth pattern, histological grade,

stromal/vascular invasion, proliferation indices and lymph node status were significantly associated with poor survival rates, whereas multivariate analysis disclosed lymph node status as the only independent prognostic factor in canine mammary tumours.

2. Alterations in the expression of the cadherin-catenin complex represent a common event in canine mammary malignant tumours, with reduced E-cadherin and β-catenin expression significantly associated with several aggressive clinicopathological features, such as high histological grade and invasion, as well as with poorer survival times. P-cadherin was only associated with invasion. Cadherin-mediated cell adhesion molecules might play a central role in canine mammary tumour progression and they may be of prognostic value in canine malignant mammary tumours.

3. EGFR is consistently expressed by canine mammary myoepithelial cells. Yet, it is also expressed by epithelial cells, being significantly associated with malignancy. EGFR expression was only associated with animal age and tumour size variables. Female dogs affected by EGFR-overexpressing malignant tumours showed poorer survival rates compared to dogs harbouring EGFR negative tumours, but the differences observed failed to reach statistically significant levels.

4. The reduction or absence of luminal CK19 expression was found significantly associated with several clinicopathological variables, such as invasion, high histological grade, high proliferative index, as well as with the expression of basal and/or myoepithelial cell markers. Univariate analysis revealed that the reduction of CK19 was significantly associated with poor survival rates, but multivariate analysis failed to confirm CK19 as an independent prognostic variable in canine mammary tumours.

5. By using a surrogate panel of immunohistochemical cell markers (which included ER, HER-2, CK5, P63 and P-cadherin), a novel human classification was applied on canine mammary carcinomas, revealing similar molecular subtypes to the ones found in human breast cancer (luminal A, luminal B, basal-like and HER-2 overexpressing), associated with distinct clinical behaviours. Basal-like phenotype was significantly associated with poor prognostic features, such as high histological grade and proliferation, as well as with shorter disease-free and overall survival rates.

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