DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

CHARACTERIZATION OF CANCER STEM-LIKE PROPERTIES IN CANINE MAMMARY CANCER

Daniela Sofia Machado Pereira

2012

DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

CHARACTERIZATION OF CANCER STEM-LIKE PROPERTIES IN CANINE MAMMARY CANCER

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Adelina Maria Gaspar Gama Quaresma (Universidade de Trás-os-Montes e Alto Douro) e da Professora Doutora Joana Cancela de Amorim Falcão Paredes (Instituto de Patologia e Imunologia Molecular da Universidade do Porto)

Daniela Sofia Machado Pereira

2012

Aos meus Pais

Aos meus irmãos

Aos meus sobrinhos

Ao Fernando.

AGRADECIMENTOS

Durante a prossecução deste meu projecto muitas foram as pessoas que directa e indirectamente, de forma abnegada e autêntica, me ajudaram e apoiaram. A elas o meu profundo reconhecimento e sincero agradecimento:

À Professora Doutora Adelina Gama, orientadora de tese de Mestrado, pela sua excelente orientação marcada pela sapiência, pela sua inesgotável e atempada disponibilidade, pelo seu brilhantismo científico, técnico e humano, enfim por tudo aquilo que é e foi durante estes anos.

À Professora Doutora Joana Paredes, co-orientadora da tese de Mestrado, pela sua inexcedível competência, excelência, por ter sido um porto seguro em momentos difíceis e uma fonte profícua de saber. Foi óptimo poder ter partilhado este ano e crescer como profissional e pessoa ao teu lado. Muito obrigado por me teres acolhido.

À Professora Doutora Anabela Alves, Professora Doutora Fernanda Travassos, Professora Doutora Maria Pinto e Professora Doutora Maria Pires, do Laboratório de Histologia e Anatomia Patológica, pela simpatia, paciência e ajuda.

Ao Professor Doutor Fernando Schmitt, exemplo de paixão pela ciência, conhecimento e dinamismo, que em muito contribuiu para o meu desempenho científico.

Ao IPATIMUP na pessoa do Professor Doutor Manuel Sobrinho Simões, por me ter aberto as portas de um exemplo cabal do que se faz bem neste país, sendo um local de referência na investigação em Oncobiologia no panorama nacional e mundial.

À Coordenadora do Mestrado em Biologia Celular e Molecular da Universidade de Coimbra, Professora Doutora Emília Duarte, pela amizade, pelos conselhos e pela forma empenhada como coordena o referido mestrado.

Às Senhoras D. Lígia Lourenço, D. Ana Plácido, D. Glória Milagres, do Laboratório de Histologia e Anatomia Patológica, pela amizade, disponibilidade, simpatia e colaboração.

Ao Sr. Oliveira, Sr. Mário, Sr. Mendes, Cátia e Zezinha pela dedicação, amizade e boa disposição.

Ao grupo de Patologia Mamária e aos restantes membros da Genética do Cancro por fazerem das longas caminhadas curtos passeios, por me fazerem acreditar que vale a pena mesmo nos dias mais difíceis, por estarem e por serem… Sem vocês dificilmente teria o mesmo sabor. Por tudo isto aqui fica gravado o meu profundo reconhecimento: André (o meu mentor), Mádá (cumplicidade), Babi (a minha protectora), Ritinha (o meu pronto socorro), Diana (de todas as horas), Ana Sofia (o poço de conhecimento), Maria Rita (doçura), Nair (simpatia), André Albergaria (pela disponibilidade); Carlinhos (boa disposição), Dani (pelos abraços de grupo), Mafalda e Inês (as companheiras), Patrícia Oliveira (a doida), João Vinagre, Bé, Adélia, Dina, Renata, Cecília, Ângela, Joaninha, Rui, Hugo, Patrícia Carneiro, Miguel, Soraia, Xu, Joana Carvalho e Catarina Leitão (IBMC).

Aos meus pais, Porfírio Pereira e Amélia Machado, que em todos os momentos, onde quer que esteja, me apoiam incondicionalmente, que me fazem sentir a importância das pequenas coisas, e que possibilitam toda a minha vivência académica.

Aos meus tios, Aninhas e Eduardo, e aos meus primos, Teresinha e Hugo por terem estado ao meu lado quando precisei, pela amizade e carinho.

Aos meus irmãos, Sónia, Bela e Porfírio, e à minha cunhada Joana, pela paciência e carinho, pelo estímulo e pela presença constante e efectiva na minha caminhada.

Aos meus sobrinhos, Samuel, Carlota e Tomás, pela alegria e amor, e por me mostrarem as coisas boas da vida, tornando-me todos os dias uma pessoa melhor.

Aos meus amigos Sílvia, Nuno, Maria, Quintas, Isa, Sara, Ana Raquel, Bibi, Ricardo, Zé Carlos, Sónia, Ritovsky, Maria João, Sofia, Ismael, Sérgio, Catarina, Sabrina, Rocha, Marisa, Edgar, Dinis, Marco, Rui, Nuno Machado, Sandra, Félix e Irene pela partilha, dedicação, amizade, apoio, alegria e companheirismo.

Ao Fernando, pelo apoio incondicional, pelos bons conselhos, pelo crescimento paralelo, compreensão, paciência, ajuda, alegria, amor e por tudo o que significa na minha vida.

ABSTRACT

One of the most motivating concepts that is being explored in the human cancer research field is the cancer stem cell (CSC) hypothesis, which states that a minority of transformed cells, with acquired stem or progenitor properties, are the source of tumour cell renewal and thereby determine tumour behaviour. In canine mammary cancer (CMC), few studies have focused on the existence of cancer stem cells. If stem/progenitor cells are the targets for transforming events in canine mammary gland, as proposed for human breast cancer, the elucidation of the molecular pathways that regulate self-renewal activity of cancer stem cells and their interaction with the microenvironmental niche will provide potential therapeutic targets for this disease.

Through an *in vitro* approach (three mammary tumour cell lines), we have identified cells with stem-like properties, by using a mammosphere formation assay. A phenotypical characterization by flow cytometry, immunofluorescence and western blot has also been performed, as well as functional assays. In addition, we have evaluated the immunohistochemical expression of CSC markers (ALDH1, CD24, CD44) in a series of 112 canine mammary carcinomas.

Our results showed that these cell lines reflect the heterogeneity of canine mammary cancer, as they display different phenotypical characteristics as well as functional properties. With regard to CSC phenotype, cell lines exhibited distinct expression patterns. Flow cytometry showed that CMT-3p, the most invasive cell line, was highly enriched for the $CD44^{+}/CD24^{low}$ CSC phenotype. All cell lines presented ALDH activity and were able to grow as spheroids in anchorage-independent conditions, with $CD44^{+}CD24^{-/low}ALDH1^{+}$ phenotype apparently related with this capacity. On the other hand, in our *in vitro* model, epithelial mesenchymal transition (EMT) was not necessarily associated with stem-cell traits, given that CMT-2p, the cell line that presented more mesenchymal markers, was the cell line that expressed less CSC markers. Concerning to canine mammary carcinomas, CD44 (81.1%) was the CSC marker most frequently observed, being rarely found in solid carcinoma subtype. CD24 was infrequent, while ALDH1 expression was frequently observed both in epithelial and stromal cells. No association was observed between CSC markers expression and aggressive tumour behaviour. In fact, a positive epithelia ALDH1 expression was significantly associated with better overall and disease-free survival.

Our results further consolidate the stem cell theory in this animal model; however, additional studies are required in order to unravel its biological significance in this complex disease.

Keywords: dog, cell lines, mammary tumours, cancer stem cells, ALDH1, CD44 and CD24.

RESUMO

Um dos conceitos mais motivantes na área da Oncobiologia é a teoria das células estaminais tumorais (CSC), a qual defende que uma minoria de células transformadas, com propriedades estaminais, constituem a origem da renovação celular neoplásica, pelo que são determinantes no seu comportamento biológico. No cancro de mama canino (CMC) são poucos os estudos acerca da existência de CSC. No entanto, caso as células estaminais/progenitores sejam de facto o alvo da transformação neoplásica na glândula mamária canina, como foi proposto para o cancro de mama humano, a elucidação das vias moleculares que regulam a actividade das CSC e a sua interacção com o microambiente, fornecerá potenciais alvos terapêuticos para esta doença.

Usando uma abordagem *in vitro* (três linhas celulares mamárias caninas), identificámos células com propriedades estaminais, através do ensaio de formação de mamosferas. Foi também realizada caracterização fenotípica por citometria de fluxo, imunofluorescência e western blot, assim como estudos funcionais. Foi ainda avaliada, pela técnica da imuno-histoquímica, a expressão de marcadores de CSC (ALDH1, CD24, CD44) numa série de 112 casos de CMC.

Os nossos resultados referentes às linhas celulares parecem refletir a heterogeneidade verificada nos tumores mamários caninos, visto apresentarem diferentes características fenotípicas e propriedades funcionais. No que diz respeito ao fenótipo estaminal, as linhas celulares apresentaram padrões de expressão diferentes. A citometria demonstrou que a linha CMT-3p, a mais invasiva, apresentou enriquecimento no fenótipo $CD44^{+}/CD24^{low}$. Todas as linhas celulares apresentaram actividade da ALDH e foram capazes de crescer em condições independents de ancoragem, como esferóides, estando o fenótipo $CD44^{+}CD24^{-/low}ALDH1^{+}$aparentemente relacionado com esta capacidade. Por outro lado, no nosso modelo *in vitro*, a transição epitelial-mesenquimatosa não parece estar associada a características de células estaminais, uma vez que a linha celular que apresentou mais marcadores mesenquimais foi a que patenteou menos marcadores de CSC.

No que diz respeito aos carcinomas mamários caninos, o CD44 (81,1%) foi o marcador observado mais frequentemente, sendo incomum nos carcinomas sólidos. O CD24 apresentou expressão rara, enquanto a expressão da ALDH1 foi observada frequentemente tanto nas células epiteliais como estromais. Não se observou qualquer associação entre o fenótipo CSC e um comportamento biológico mais agressivo. Pelo contrario, a expressão da ALDH1 encontrou-se significativamente associada com melhor prognóstico.

Os nossos resultados reforçam a teoria das CSC neste modelo animal; no entanto, consideramos essenciais estudos adicionais *in vitro* e *in vivo* no sentido de descortinar o seu significado biológico nesta complexa doença.

Palavras-chave: cão, linha celular, tumores mamários, células estaminais tumorais, ALDH1, CD44, CD24.

PUBLICATIONS

The present results have been partially presented or accepted for presentation:

- Pereira D, Vieira AF, Schmitt F, Paredes J, Gama A (2012). Identification and characterization of a stem cell population in canine mammary cancer, V Scientific Journeys of Biochemistry, UTAD-Vila Real, 29th March, 2012 (Oral presentation).
- Pereira D, Vieira AF, Schmitt F, Paredes J, Gama A (2012). Looking for ALDH expression and activity in canine mammary tumours and cell lines, 7th International Annual Meeting of the Portuguese Society for Stem Cells and Cell Therapies, Oporto, 26-27th April 2012 (Poster presentation).
- Pereira D, Vieira AF, Schmitt F, Paredes J, Gama A (2012). Targeting cancer stem cells in canine mammary cancer**,** 30th Meeting of the European Society of Veterinary Pathology, Léon, 5th-8th September 2012 (Accepted for oral presentation).

TABLE OF CONTENTS

ABBREVIATIONS

AKT	Protein kinase B
ALDH1	Aldehyde dehydrogenase 1
APC	*Adenomatous Polyposis Coli*
APC	Allophycocyanin
BAAA	BODIPY®- aminoacetaldehyde
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BrdU	Bromodeoxyuridine
BSA	Bovina serum albumin
CD	Cluster of differentiation
CD24	Cluster of differentiation 24
CD44	Cluster of differentiation 44
CK	Cytokeratin
CMC	Canine mammary cancer
CMT	Canine mammary tumours
CSC	Cancer stem cell
DAPI	4,6-diamidine-2-phenylindolendihydrochrolide
DEAB	Diethylaminobenzaldehyde
DFS	Disease-free survival
Dhh	Desert hedgehog
DNA	Deoxyribonucleic acid
DSH	Disheveled protein
E-cad	Epithelial cadherin
ECM	Extracellular matrix
EDTA	Etilenediaminetetracetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal-inactivated bovine serum

Fib	Fibronectin
FITC	Fluorescein isothiocyanate
FZD	Frizzled
GPI	Glycosil phosphatidylinositol
GSK3β	Glycogen synthase kinase-3β
HE	Haematoxilin and eosin
HER2	Human epidermal growth factor receptor 2
Hh	Hedgehog
HSA	Heat stable antigen
IDC-NOS	Invasive ductal carcinomas not otherwise specified
IHC	Immunohistochemistry
Ihh	Indian hedgehog
L1	Glycan-binding adhesion molecule
Lin	Lineage
LRP	LDL-receptor-related protein
MDCK	Madin-Darby canine kidney
MET	Mesenchymal to epithelial transition
MFE	Mammosphere forming efficiency
MMP	Matrix metalloprotease
N-cad	Neural cadherin
NICD	Notch intracellular domain
NOD-SCID	Non-obese diabetic/severe combined immunodeficiency
OS	Overall survival
PBS	Phosphate buffered saline
P-cad	Placental cadherin
PE	Phycoerythrin
PgR	Progesterone receptor
PI3K	Phosphatidylinositol-3-kinase
Ptch1	Patched
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Shh	Sonic Hedgehog
siRNA	Small interference RNA

SMA	Smooth Muscle Actin
Smo	Smoothened
TGFβ	Transforming growth factor beta
TNBC	Triple-negative breast cancer
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
Vim	Vimentin
WHO	World Health Organization
α6-INT	Alpha-6 integrin
α-SMA	Alpha-smooth muscle actin
α-tub	Alpha tubulin
β4-INT	Beta-4 integrin
β-act	Beta actin
β-cat	Beta catenin

INTRODUCTION

1. CANINE MAMMARY TUMOURS

Canine mammary gland, similarly to the one from felines, rodents and humans, is frequently affected by spontaneous tumours. These tumours constitute approximately 25 to 50% of all neoplasias in female dog, occurring in a frequency three times greater than the level reported in human populations (Strandberg and Goodman, 1974, Misdorp et al., 1999, Sorenmo, 2003, Oliveira et al., 2009). Canine mammary tumours represent a heterogeneous group in terms of morphology and biological behaviour, and have been the focus of intensive research over the last few decades (Gama, 2011). These tumours constitute a serious problem in worldwide veterinary practice and a matter of concern for both oncologists and pathologists. Canine mammary cancer has been suggested as an excellent model for studying human breast cancer, due to their similar histologic origin and comparable regional and systemic metastasis. In addition, these appear in significant numbers at a similar relative age (Strandberg and Goodman, 1974, Destexhe et al., 1993, Munson and Moresco, 2007).

Dogs usually develop five pairs of mammary glands, although four or six pairs have been found in a few animals. There are two thoracic (M1 and M2), two abdominal (M3 and M4), and one inguinal (M5) pair of mammary glands and each teat has between seven and sixteen duct openings. Each duct will constitute a lobe of the adult mammary gland, acting as an independent functional unit within the gland. During dog development and growth, mammary glands continue to develop and, until the time of puberty, the ducts extend only a short distance from the teat (Sorenmo et al., 2011).

In the normal canine and human mammary glands, ducts and lobules are lined by 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 and morphologically heterogeneous, since these cells appear either spindle-shaped or cuboidal, depending on their location in the branching structure of breast ducts and on the hormonal status of the tissue (Gusterson et al., 2005, Sorenmo et al., 2011). In both species, the luminal epithelial cells have been identified with markers for epithelial cells, being characterized by the expression of low molecular weight luminal cytokeratins (CKs), including CK8, CK18, CK19, and CK7 (Destexhe et al., 1993, Foulkes, 2004, Gusterson et al., 2005, Gama et al., 2010, Goldschmidt et al., 2011, Sorenmo et al., 2011). In contrast, the outer cell layer is formed by cells that variably express high molecular weight basal CKs, such as CK5, CK6, CK14 and 17, in addition to other markers such as alpha-smooth muscle actin (α-SMA), calponin, p63, P-cadherin and vimentin (Destexhe et al., 1993, Gama et al., 2003, Gama et al., 2004, Gusterson et al., 2005, Gama et al., 2008b, Reis-Filho and Tutt, 2008, Gama et al., 2010, Sassi et al., 2010, Goldschmidt et al., 2011, Sorenmo et al., 2011).

In veterinary medicine, mammary gland tumours are classified according to their morphology, providing good prognostic indications that may be enhanced by further prognostic tools, such as staging, histological grade, tumour size, proliferation indexes, lymph node status, hormone receptor status and adhesion molecules expression. However, canine malignant mammary tumours are a heterogeneous group of neoplasms, which would greatly benefit from a classification system that addresses molecular differences, similar to those described for breast cancer in women (Sorlie et al., 2001). Based on this molecular classification, a few studies have also defined different subgroups in canine mammary cancer, namely luminal and basal-like cancers (Sorlie et al., 2001, Gama et al., 2008a, Sassi et al., 2010). Only Gama et al. described a HER2 overexpressing subgroup (Gama et al., 2008a).

Griffey *et al*. (1993) applied the "basal carcinoma" nomenclature in canine mammary carcinomas for the first time, based on the immunohistochemical expression of CK14; like in humans, these carcinomas showed aggressive clinical behaviour, characterized by the lack of ER, PgR and HER2 expression, leading to an association with triple-negative breast cancer (TNBC). Interestingly, Gama *et al.* (2008) found, using the canine model, an association between basal-like molecular subtype with specific carcinoma tumour histological types (like simple carcinoma and carcinosarcoma types), and with carcinomas harbouring high histological grade, lymphovascular invasion, high proliferation and low survival rates, corroborating the results described in recent human literature for basal-like breast cancer (Griffey et al., 1993, Matos et al., 2005, Kim et al., 2006, Livasy et al., 2006, Rakha et al., 2006, Gama et al., 2008a, Sassi et al., 2010).

It is not known if basal-like phenotype represents a signature derived from the cell of origin of these cancers, or if it is the result of differentiation from a precursor that is common to all breast cancers and, therefore, does not reflect histogenesis. Some authors have been proposing that there is a close relationship between the basal/myoepithelial compartment and the stem/progenitor cells of the mammary gland, postulating a "hierarchy or stem cell" model of breast carcinogenesis to elucidate the observed functional heterogeneity of breast tumours (Dick, 2003, Polyak and Hu, 2005, Constantinidou et al., 2010, Sorenmo et al., 2011). Polyak (2007) suggested that there are distinct tumour progression pathways for each tumour type, proposing two hypothetical models to explain the differences among distinct molecular subtypes (Figure 1) (Polyak, 2007b).

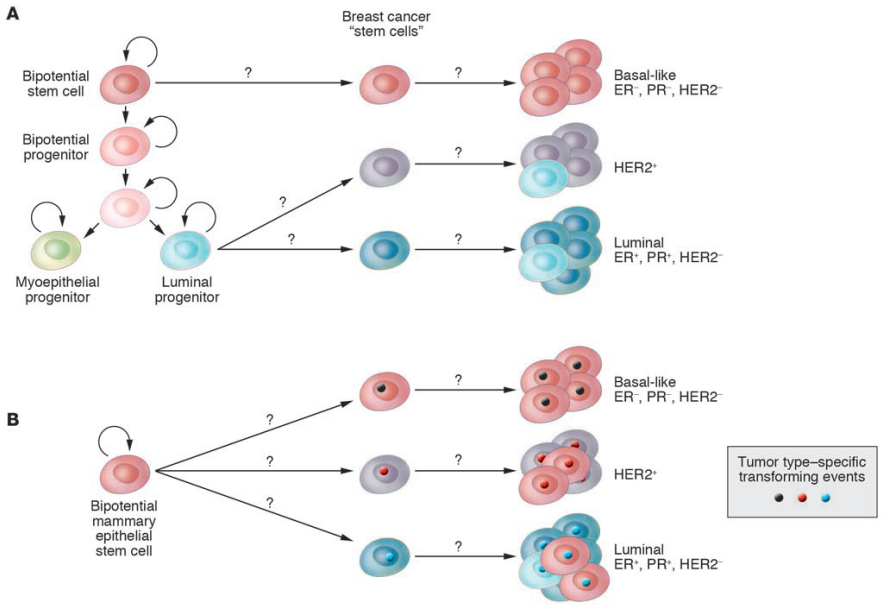


Figure 1. Breast tumour molecular subtypes explained by two hypothetical and distinct models. (A) Based on the cell of origin model, each tumour subtype is initiated in a different cell type from the normal differentiation hierarchy of the breast (presumably stem or progenitor cell). (B) Based on the tumour subtype-specific transforming event model, in which the cell of origin can be the same for different tumour subtypes, the tumour phenotype is primarily determined by acquired genetic and epigenetic events (adapted from Polyak, 2007b).

2. CANCER STEM CELLS (CSCS)

Stem cells have been proposed as an attractive target for cancer transformation, since they share many characteristics with cancer cells, including the mechanisms that regulate self-renewal. In addition, the notion that tumours contain CSCs, which are rare cells with indefinite proliferative potential that drive tumour formation and growth, also support this hypothesis. Indeed, these cells are commonly defined by their *in vivo* tumour initiation capacity. Correlating with this, several pathways and genes required for normal stem cell function are activated in CSCs and play essential roles in tumorigenesis (Reya et al., 2001, Shipitsin et al., 2007, Gadalla et al., 2011).

Like normal stem cells, CSCs can divide either symmetrically or asymmetrically. In some circumstances, a stem cell can be stimulated to divide symmetrically, to produce two stem cells, which lead to an increase of the stem cells pool. These stem cells are considered to be immortal, proliferating indefinitely until they are induced to differentiate or to go under apoptosis. But, curiously, asymmetric DNA segregation during cell division is a property only ascribed to stem and progenitor cells. In this type of division, one daughter cell maintains stemness like its mother cell,

while its sib, a progenitor cell, can start its role as a potential origin of a differentiated cell lineage, as well as the transit cell to expand the population of that particular lineage (Trosko, 2009).

In humans, cancers of the hematopoietic system (leukaemias) provide the best evidence that normal stem cells are the targets of transforming mutations and that cancer proliferation is driven by CSCs. In this particular disease, both normal stem cells and CSCs are well characterized (Reya et al., 2001). However, it has been shown that breast cancer is also maintained by a set of relatively few cells termed breast cancer tumour initiating cells or CSCs. These small number of human breast cancer cells are able to initiate tumour formation upon transplantation into the cleared mammary fat pad of NOD-SCID mice (Stingl and Caldas, 2007, Gadalla et al., 2011).

In recent literature, it has been shown that CSCs are not only defined as a subset of tumour cells with stem-cell-like properties, but are thought to be responsible for the growth, progression, drug resistance, tumour recurrence, fast blood vessel formation, promotion of cell motility and metastasis (Polyak, 2007a, Shipitsin et al., 2007, Charafe-Jauffret et al., 2008). Thus, it is important to note the difference between CSCs and the tumour cell of origin. The last is a normal cell that acquires the first cancer-promoting mutations, also known as cancer-initiating cells. However, this is not necessarily related to CSCs, which constitute a cellular subset within the tumour that uniquely sustains malignant growth, also known as cancer-propagating cells (Figure 2) (Visvader, 2011).

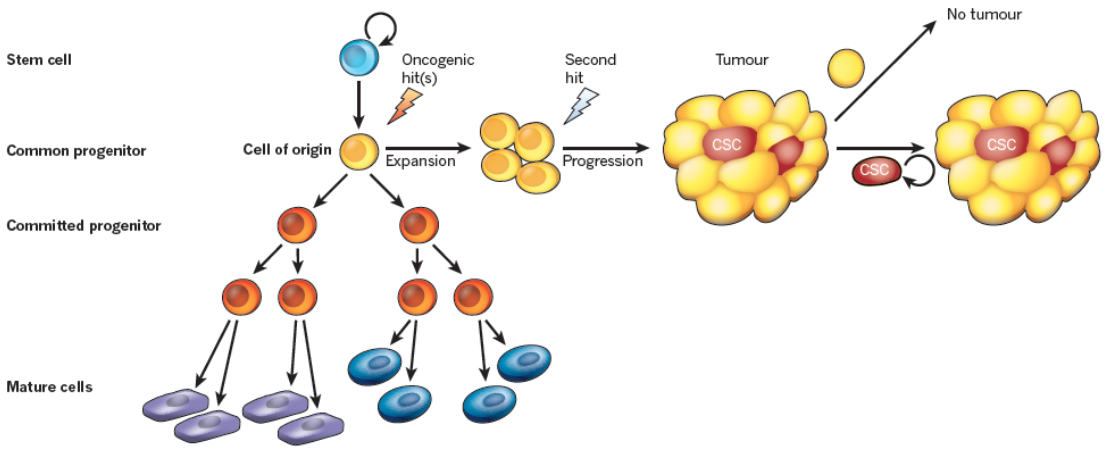


Figure 2. The cell of origin and evolution of a CSC. The cell of origin for a particular tumour could be an early precursor cell, such as a common progenitor; the accumulation of further epigenetic mutations by a cell within the aberrant population (in this case expanded) during neoplastic progression may result in the emergence of a CSC. In this model, only the CSCs (and not other tumour cells) are capable of sustaining tumorigenesis. Thus, the cell of origin, in which tumorigenesis is initiated, may be distinct from the CSC, which propagates the tumour (adapted from Visvader, 2011).

2.1. CSC Hypothesis

Two distinct models were proposed to explain the establishment and maintenance of tumour heterogeneity (Figure 3). Although not necessarily mutually exclusive, the suggested theories are: 1) the clonal evolution model (or stochastic theory) and 2) the CSC hypothesis (or hierarchy theory). The combination of these two models (e.g., clonal evolution of CSCs) was also proposed as plausible (Dick, 2003, Campbell and Polyak, 2007, Polyak, 2007b, Visvader and Lindeman, 2008).

Portraying the clonal evolution model, tumour cell phenotypes are determined based on the combination of the cell type of origin and the acquisition of various combinations of genetic mutations, epigenetic alterations, and paracrine signals from surrounding cells. It has been proposed that genetic drift and stepwise natural selection for the fittest, most aggressive cells drive tumour progression. According to this, tumour initiation is caused by multiple alterations that occur in a random single cell, providing it with a selective growth advantage over adjacent normal cells. Cellular phenotypes are not stable during this process and can change as the tumour evolves. This genetic instability and uncontrolled proliferation can allow the production of cells with additional mutations and hence, new characteristics. These cells may leave a large number of offspring by chance, or the new mutations may provide a growth advantage over other tumour cells, such as resistance to apoptosis. In either case, new subpopulations of variant cells are born, and other subpopulations may contract, resulting in tumour heterogeneity. In other words, according with this model, all cells have a high proliferative capacity, and are also capable to undergo self-renewing division; thus, all these cells have the potential to contribute to tumour progression and drug resistance (Miller et al., 2005, Polyak, 2007b).

In the present work, we will be focusing on the CSCs hypothesis, which states that there is a particular fraction of cells resident within the tumour with stem cell-like properties, called CSCs, which drive tumour initiation, progression, and recurrence (Dick, 2003, Wicha et al., 2006, Campbell and Polyak, 2007, Polyak, 2007b, Visvader, 2011). Lindeman and Visvader (2010) referred to CSCs as the apex of the tumour hierarchy, analogous to stem cells in the normal epithelial hierarchy, which do not necessarily need to have arisen through the transformation of a normal stem cell (Dick, 2003, Lindeman and Visvader, 2010, Monroe et al., 2011). However, Campbell and Polyak (2007) reported that CSCs are widely believed to arise from normal stem or progenitor cells of an adult tissue (Campbell and Polyak, 2007).

Indeed, many theories in biology are supported by many general observations. First of all, normal stem cells may be the cells in which cancer begins, because they have been shown to exist in many tissues from which cancer often develops, such as blood, brain, lung, breast and prostate

(Campbell and Polyak, 2007). These cells are also long-living, suggesting that are more susceptible to acquire multiple mutations/alterations than other cells (Campbell and Polyak, 2007, Charafe-Jauffret et al., 2008). Second, the tumour traits of monoclonality, unlimited proliferative capacity, and phenotypic heterogeneity that includes a variety of differentiation states with some non-dividing cells, could be explained by tumours originating from a self-renewing, multipotent, and slow cycling cell (Miller et al., 2005). Third, the epigenetic mechanisms and the extracellular microenvironment can regulate both normal stem cells and cancer cells, which regulate several cellular functions, such as induction of angiogenesis, resistance to apoptosis and drugs, and cell migration, associating stem-like cancer cells with tumour initiation, recurrence, and metastasis (Reya et al., 2001, Visvader, 2011). In addition, cancer cells often show signalling pathways normally associated with stem cell function, such as Wnt, Hedgehog, and Notch pathways, either activated or deregulated (Hill, 2006). Finally, the observation that a large number of tumour cells are required to successfully transplant tumours, even between syngeneic mice with identical immune systems, suggests that only a fraction of tumour cells are tumorigenic (Hill, 2006, Wicha et al., 2006).

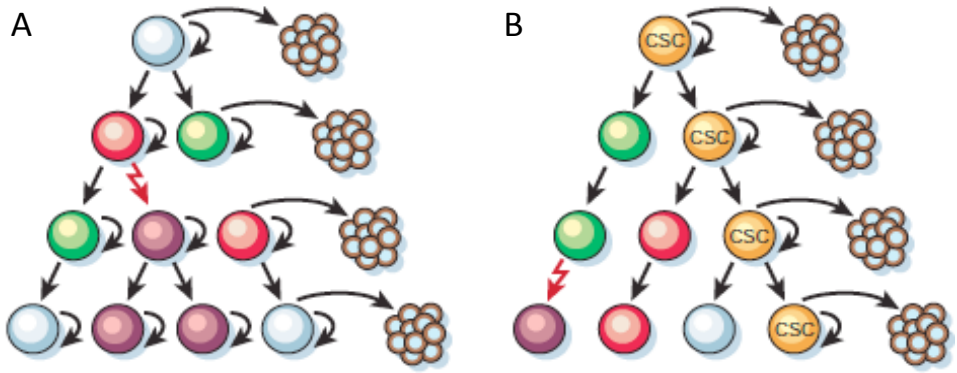


Figure 3. Two general models to explain heterogeneity in solid cancers. (A) In the clonal evolution model, all cells have similar tumourigenic capacity. New tumourigenic clones may emerge in a stochastic manner and contribute to tumour heterogeneity. (B) In the CSC model, subsets of CSCs are capable of tumour propagation. These cells have self-renewal properties and are capable of recapitulating a tumour hierarchy (adapted from Reya et al., 2001).

2.2. Breast CSC Markers

The main goal of both researchers and oncologists is to understand how many and which tumour cells must be eliminated for a given treatment to succeed. Taking into account the new concept of CSCs, this can be used to explain why current cancer therapies (chemotherapy and radiation) are relatively ineffective (Sheridan et al., 2006, Charafe-Jauffret et al., 2009a, Trosko, 2009, Resetkova et al., 2010).

Considering the CSC hypothesis, it is important to identify and eliminate CSCs, in order to eradicate the tumour. Thus, expression of specific cell surface markers has been widely used to isolate CSCs, but these markers vary depending on tissues or species. The following markers have been considered as the ones important to study human breast CSCs.

2.2.1. CD44/CD24

Putative stem and differentiated epithelial cells have been purified from cancerous and normal breast tissue, using the cell surface markers CD44 and CD24 (Polyak, 2007b).

CD44, also named Hermes antigen, H-CAM or Pgp-1 antigen, is one of the most well recognized breast CSC markers and is a widely distributed integral membrane glycoprotein expressed by a variety of normal and neoplastic cell types, being involved in cell-cell and cell-matrix interactions. It is a known receptor for hyaluronic acid and interacts with other ligands, such as matrix metalloproteases (MMPs) (Ohene-Abuakwa and Pignatelli, 2000, Al-Hajj et al., 2003, Monroe et al., 2011). CD44 is a molecule with multiple isoforms, with pleiotropic roles in cell signalling, proliferation, adhesion, migration and homing, whose expression has been reported to allow selective enrichment for breast tumour-initiating cells (Raouf et al., 2008, Visvader and Lindeman, 2008, Brown et al., 2011). Approximately twenty CD44 isoforms have been described, which are the result of alternative RNA splicing, occurring from a single gene found on the short arm of the human chromosome 11. In contrast to non-neoplastic tissues, at least nine alternative spliced products of the CD44 gene showed amplified expression in breast and colonic carcinomas and their metastasis. The standard isoform CD44H or CD44s (90kDa) is broadly distributed in haematopoietic cells, fibroblasts, and numerous tumours of both mesenchymal and neuroectodermal origin, whereas CD44E (150kDa) appears restricted to subsets of normal epithelial cells. Curiously, CD44v is expressed by several malignant cells, being described in human cancers such as melanomas, lung cancers and brain metastasis, with its expression correlating with high metastatic potential (Ohene-Abuakwa and Pignatelli, 2000, Visvader and Lindeman, 2008).

CD24, also known as heat stable antigen (HSA) in mouse, consists in a small protein comprising 27 amino acids, which is extensively glycosylated and is bound to the membrane via a glycosyl phosphatidylinositol (GPI) anchor of heterogeneous molecular weight (ranging from 30 to 70kDa) (Aigner et al., 1997, Kristiansen et al., 2003, Baumann et al., 2005). This protein functions as an adhesion molecule for P-selectin and L1 and is expressed in cells of the hematopoietic system, such as at the early stages of B-cell development and neutrophils, in neuronal tissue, and in certain epithelial cells, such as keratinocytes and renal tubular epithelium. However, it is absent

on normal T cells or monocytes (Aigner et al., 1997, Baumann et al., 2005, Visvader and Lindeman, 2008).

Although CD24 is not usually found in adult human tissues, it is expressed in many carcinomas and it is thought that its expression is related with tumorigenesis, progression and metastasis. Polyak (2007) found an increased number of $CD24^+$ cells in distant metastases compared to matched primary tumours, possibly because CD24 has been identified as an alternative ligand of P-selectin, an adhesion receptor on activated endothelial cells and platelets (Aigner et al., 1997, Kristiansen et al., 2003, Baumann et al., 2005, Campbell and Polyak, 2007, Polyak, 2007a).

Interestingly, several studies described a subpopulation of cells in human mammary carcinomas with the phenotype $CD44^+/CD24^-/lin^-$ that display CSC properties. This evidence was first described by Al-Hajj and colleagues (2003), which showed that this cell population shares with normal stem cells the ability to proliferate extensively and to give rise to diverse cell types with reduced developmental or proliferative potential. These authors also showed that as few as 200 cells with this phenotype were able to generate tumours in NOD/SCID mice, whereas 20,000 cells that did not display this phenotype failed to do so (Al-Hajj et al., 2003, Korkaya et al., 2008, Charafe-Jauffret et al., 2009a, Bhat-Nakshatri et al., 2010).

2.2.2. ALDH1

ALDH enzymes catalyse the oxidation of aliphatic and aromatic aldehydes to carboxylic acids. Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, which have a role in early differentiation of stem cells through its role in oxidizing retinol to retinoic acid (Ginestier et al., 2007, Charafe-Jauffret et al., 2008, Visvader and Lindeman, 2008, Charafe-Jauffret et al., 2009a). Retinoic acid signalling is linked to cellular differentiation during development and plays a role in stem cell self-protection throughout an organism lifespan (Croker et al., 2009).

Interestingly, Ginestier et al. (2007) demonstrated that expression of ALDH1 in human breast tumours is a predictor of poor clinical outcome, and that high ALDH1 activity selects for both normal and tumorigenic human mammary epithelial cells with stem/progenitor properties (Ginestier et al., 2007, Croker et al., 2009, Resetkova et al., 2010). In addition, in animal models, 500 cells from the subset $ALDH1^+$ were found to give rise to tumours in as few as 40 days; this tumour forming ability of $ALDH1^+$ was further enhanced if cells shared the $CD44^+/CD24^-$ phenotype, with the cell population bearing both CSCs phenotypes being characterized by high tumourigenic capacity, generating a tumour from as few as 20 cells. In contrast, $ALDH1^-$ cells and

$CD44^+/CD24^-/Lin^-$ were not able to generate tumours, even when implanted 50000 cells/fat pad (Ginestier et al., 2007, Resetkova et al., 2010).

Therefore, the use of ALDH1 activity as a purification strategy allows non-toxic and efficient isolation of human stem-like cells. Novel findings by Croker and collaborators (2009) showed that CSC markers expression select for stem-like breast cancer cells with enhanced malignant and metastatic properties, suggesting $ALDH^{hi}/CD44^+/CD24^-$ stem-like cells as important contributors to breast cancer metastasis (Croker et al., 2009).

Interestingly, cells with the $CD44^+/CD24^-/ALDH1^+$ phenotype are more frequently found in basal-like than in luminal breast tumours, which highlights an enrichment of putative tumour-initiating cells in the aggressive basal-like subtype (Ricardo et al., 2011).

2.2.3. Integrins

Integrins represent a major family of transmembrane receptors for cell adhesion, mediating cell-ECM (extracellular matrix) and cell-cell adhesive interactions. They also regulate signal transduction pathways important for a wide variety of cell functions, such as the control of cell shape, motility, proliferation, survival and cell-type-specific gene expression (Figure 4A), playing an important role in morphogenesis, differentiation and maintenance of mammary stem and progenitor cells (Hynes, 2002, Gilcrease, 2007, Raymond et al., 2012).

These proteins are heterodimers, composed by a single α and a single β subunit through a non-covalent bound. Both subunits participate in ligand recognition, with multiple different integrins being expressed in most cells of adult mammals (Gilcrease, 2007, Raymond et al., 2012). To date, at least 8 β and 18 α subunits, which assemble into 24 different integrins with distinct ligand specificities, have been discovered (Figure 4B) (Hynes, 2002, Taddei et al., 2003, Barczyk et al., 2010). Most reports describe heterogeneous patterns of integrin expression in tumour specimens, although the role of integrins is very clear concerning the maintenance of epithelial cell polarity and the regulation of epithelial differentiation, since their ablation could result in abnormalities and lethality (Gilcrease, 2007).

Focal Adhesion Kinase (FAK) is a cytoplasmic tyrosine kinase which has been implicated in signal transduction promoted by integrins, as well as by other cell surface receptors, being linked with increased cell invasion (Luo et al., 2009, Ablett et al., 2012). Also, a direct link between integrin signalling through FAK and breast CSCs has been suggested by Luo et al. (2009), which demonstrated that FAK ablation reduced the number of CSCs in primary tumours obtained from FAK-knockout mice (Luo et al., 2009). Other studies demonstrated the importance of the integrin

signalling in cell survival by the activation of FAK and PI3K-Akt pathway, inhibiting key apoptotic molecules and activating anti-apoptotic signalling pathways (Figure 4C) (Shaw, 1999, Mercurio et al., 2001a, Gilcrease, 2007, Pontier and Muller, 2009, Zhong and Rescorla, 2011). The previous data suggest that integrins expression could mediate resistance to anoikis, an apoptotic mechanism that is induced by loss of cell anchorage (Weaver et al., 2002, Zahir et al., 2003, Carroll et al., 2006).

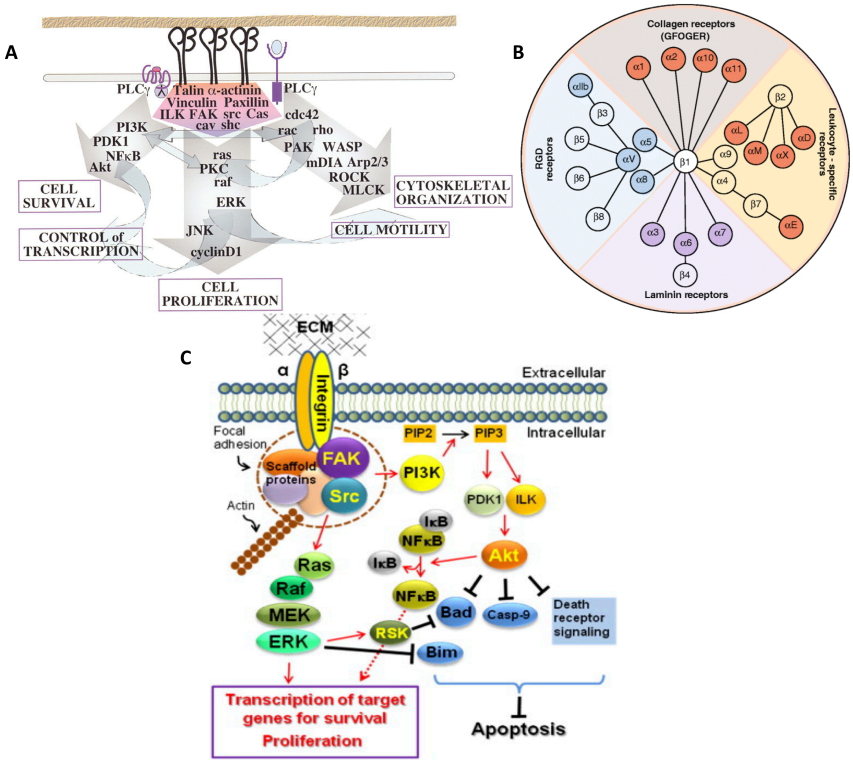


Figure 4. (A) Integrins synergized with other cell surface receptors including growth factor receptors activate largely unknown signalling pathways to affect cell proliferation and differentiation, cell shape and migration, and other events (adapted from Hynes, 2002); (B) Representation of the integrin family. In vertebrates, the integrin family contains 24 heterodimers (adapted from Barczyk et al., 2010); (C) Integrin-ECM interaction activates intracellular signalling pathways leading to cell survival (adapted from Zhong and Rescorla, 2011).

In fact, integrin receptors have been clearly implicated in human breast cancer and evidence exists demonstrating that α6β1 and α6β4 integrin heterodimers and their intracellular signalling effects play essential roles in tumour initiation and progression (Shaw, 1999, Mercurio et al., 2001b, Raymond et al., 2012). Thus, it is thought that the attachment of stem/progenitor cells through α6β1 and α6β4 to interstitial matrix components, such as laminins, might support their stem cell activity, or contribute to a cancer stem cell phenotype (Gilcrease, 2007, Pontier and Muller, 2009).

2.3. CSCs Regulation

Similar signalling pathways appear to regulate self-renewal in normal stem cells, as well as in CSCs. Indeed, it is known that a number of genetic pathways involved in stem cell self-renewal are highly involved in cancer development. These pathways, that include Hedgehog (Hh), Notch and Wnt pathways (Figure 5), have been shown to promote the self-renewal of somatic stem cells, as well as neoplastic proliferation in the same tissues, when deregulated (Pardal et al., 2003, Miller et al., 2005). Studies on these pathways have revealed close links between cancer cells and normal stem cells and uncontrolled activation of these pathways may result in specific cancers, possibly as an attempt to recapitulate normal embryonic organogenesis (Tysnes and Bjerkvig, 2007).

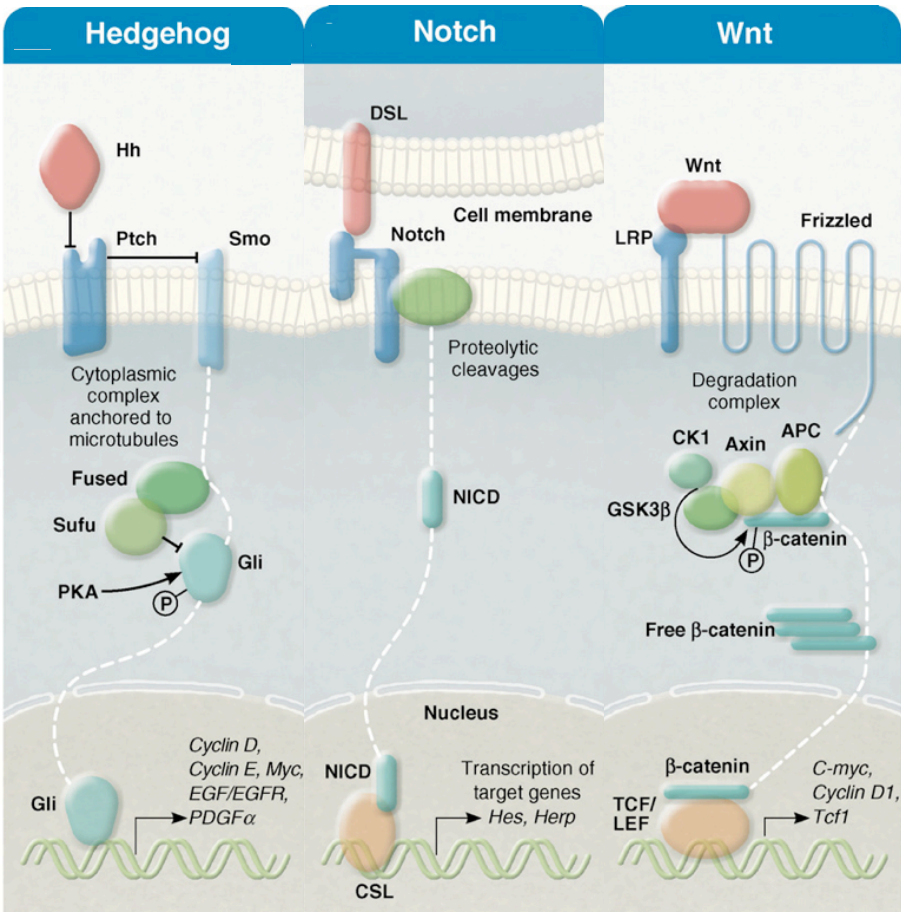


Figure 5. A simplified scheme demonstrating the major pathways associated with stem cells and cancer (adapted from Tysnes and Bjerkvig, 2007).

2.3.1. Hedgehog signalling

Three Hh homologues have been identified in vertebrates, contrasting with the single *Hh* gene found in *Drosophila*, which were called Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh), being expressed at different stages of ontogeny in different tissues and harbouring distinct biological functions. The Hh pathway directs growth and tissue patterning during embryonic development. It has been associated with self-renewal and regulation of stem cell number and tumourigenesis (Beachy et al., 2004, Liu et al., 2005, Miller et al., 2005). Hh signal transduction is initiated by the binding of the processed and lipid modified Hh ligand to its receptor Patched (Ptch1), a 12-pass transmembrane protein. In the absence of the Hh protein, Ptch1 represses signal transduction by inhibiting the Smoothened (Smo) transmembrane protein. Upon Hh binding, the inhibitory function of Ptch1 on Smo is abolished, resulting in Smo activation. The ultimate step in the pathway consists in a dissociation of the large cytoplasmic complex from microtubules and the translocation of Gli transcriptional activator to the nucleus, leading to the transcriptional activation of Hh target genes (Beachy et al., 2004, Liu et al., 2005, Miller et al., 2005, Tysnes and Bjerkvig, 2007, Kasper et al., 2009).

Studies have indicated that Hedgehog signalling is important in embryonic mammary gland induction, ductal morphogenesis and alveolar development. Genetic analyses of two hedgehog genes, Ptch1 and Gli-2, have shown that these genes have a critical role for hedgehog signalling in mediating epithelial stromal interactions during ductal development. The disruption of either genes leads to ductal dysplasia similar to the hyperplasias of the human breast. Therefore, Liu and collaborators verified the role of hedgehog signalling in mammary cell fate determination, through mammosphere-based culture system and showed that the addition of recombinant Shh can stimulate the formation of primary and secondary mammospheres and can also increase mammosphere size. These authors also used Smo inhibitor that blocked the process of mammosphere formation (Liu et al., 2005). The importance of hedgehog signalling in carcinogenesis has also been demonstrated by the fact that Ptch appear mutated in sporadic forms of cancers, which would mean that this gene can function as a tumour suppressor, by the fact that many of the genes involved in hedgehog signalling are known oncogenes, including Smo, Shh, Gli-1 and Gli-2. Additionally, the constitutive overexpression of Hh is also observed in a number of cancers (Beachy et al., 2004, Liu et al., 2005, Miller et al., 2005).

2.3.2. Notch signalling

The Notch signalling pathway is crucial in the regulation of cell fate in a variety of tissues. In mammals, Notch proteins are represented by four homologues, Notch1 to Notch4, expressed in a variety of stem or early progenitor cells. They interact with several surface-bound ligands, known as DSL (Delta, Delta-like, Jagged1 and Jagged2 in mammals), that are present on neighbouring cells. The transmembrane Notch receptors undergo proteolytic cleavage after ligand binding, releasing the Notch Intracellular Domain (NICD). This intramembrane cleavage is followed by the translocation of NICD to the nucleus, where it interacts with the DNA-binding protein CSL. The last step comprise several mechanisms, in which CSL is transformed into a transcriptional activator, and Hes and Herp Notch target genes are activated, forcing the cell to stay in an undifferentiated state (Liu et al., 2005, Glazer et al., 2007, Tysnes and Bjerkvig, 2007, Bao et al., 2011).

Depending on the cellular and development context, Notch pathway acts as a regulator of cell survival and cell proliferation. Notch signalling has been highlighted as a pathway involved in breast development and is frequently deregulated in invasive breast cancer (Dontu et al., 2004, Liu et al., 2005, Farnie and Clarke, 2007, Glazer et al., 2007). Abnormal expression of Notch receptors has been found in different types of epithelial metaplastic and neoplastic lesions, suggesting that Notch act as proto-oncogene (Dontu et al., 2004, Liu et al., 2005). In the same study, Dontu et al. (2004) concluded that this pathway may contribute to mammary carcinogenesis by deregulating the self-renewal of normal mammary stem cells (Dontu et al., 2004). Therefore, Farnie and Clarke (2007) investigated the role of Notch in pre-invasive breast lesions and found that aberrant activation of Notch signalling is an early event in breast cancer (Farnie and Clarke, 2007). Findings from Liu et al. (2005) suggested that Notch signalling is active in several distinct developmental stages of the mammary gland and acts as a regulator of asymmetric cell fate decisions (Liu et al., 2005).

2.3.3. Wnt signalling

Wnt signalling, initially identified in early embryogenesis of *Drosophila*, is involved in a large set of cellular processes, including proliferation, differentiation, migration, and apoptosis (Benhaj et al., 2005, Suzuki et al., 2008). Wnt proteins are secreted molecules that regulate proliferation and patterning during development, being also important in stem cell lineage determination and homeostasis in a variety of tissues, including the mammary gland (Pardal et al., 2003, Liu et al., 2005, Miller et al., 2005).

So far, the most well characterized Wnt signalling pathway is called the canonical Wnt pathway. This pathway involves the binding of a soluble Wnt protein to Frizzled (FZD) and LDL-receptor-related protein (LRP5 or LRP6) transmembrane receptors, leading to their binding to the intracytoplasmatic proteins disheveled (DSH) and axin, respectively. DSH will inhibit the activity of a multiprotein complex that includes the *adenomatous polyposis coli* (APC), glycogen synthase kinase-3β (GSK3β) and the aforementioned axin. The normal function of this complex is to phosphorylate β-catenin (β-cat) and thereby target it for ubiquitination and proteolysis. Wnt signalling inhibits β-cat destruction, leading to accumulation and translocation of β-cat to the nucleus where it can activate several target genes associated with cell proliferation and self-renewal (Brennan and Brown, 2004, Miller et al., 2005, Tysnes and Bjerkvig, 2007).

Due to its importance, the Wnt pathway is implicated in the pathogenesis of a number of cancers, usually by activating mutations that result in a ligand-independent state of constitutive activity (Beachy et al., 2004, Miller et al., 2005). A direct role for dysfunction of this pathway in cancer was established by experiments in transgenic mice that showed that activation of the Wnt signalling pathway in epidermal stem cells leads to epithelial cancers. Moreover, a role for Wnt signalling in self-renewal of mammary stem cells was suggested by some studies, and this may imply that mammary stem cells and progenitor cells might be targets for oncogenesis by Wnt (Brennan and Brown, 2004, Liu et al., 2005).

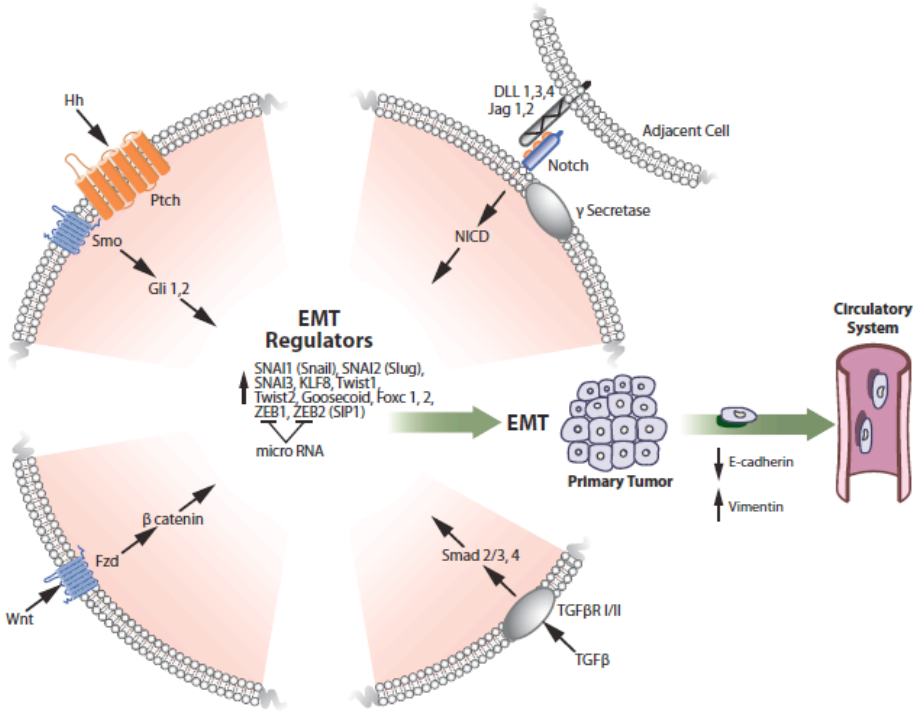


Figure 6. Several pathways leading to Epithelial-Mesenchymal Transition (adapted from Takebe et al., 2011).

3. CSCs AND EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

Several studies have suggested that the different pathways involved in the regulation of stem cells self-renewal could play a key role not only in the regulation of CSCs, but also in the Epithelial-Mesenchymal Transition (EMT) during tumour progression (Figure 6) (Bao et al., 2011, Takebe et al., 2011), raising a possible link between CSCs and EMT.

In fact, breast CSCs are increasingly thought to play a major role in breast cancer growth and formation of metastasis and recent observations imply that there is a crosstalk between EMT and CSC properties (Mani et al., 2008, Creighton et al., 2010, Hayashida et al., 2011, Li and Zhou, 2011, Pang et al., 2011, Takebe et al., 2011).

EMT is a morphogenetic program crucial for epithelial plasticity during embryogenesis, wound healing and tissue homeostasis. During this process, epithelial cells are converted in mesenchymal cells through profound disruption of cell-cell junctions and extensive reorganization of the actin cytoskeleton. Therefore, this process is characterized by loss of expression of many markers of differentiation, acquisition of fibroblastic-like properties, reduced intercellular adhesion and increased motility (Figure 7) (Morel et al., 2008, Radisky and LaBarge, 2008, Kalluri and Weinberg, 2009, Lindley and Briegel, 2010, Brown et al., 2011, Li and Zhou, 2011). EMT has also been recognized not only as a physiological mechanism for development and tissue remodelling, but also as a pathological mechanism in the progression of various diseases including inflammation, fibrosis and cancer (Li and Zhou, 2011).

Cell motility is a fundamental aspect to early cancer metastasis, and EMT can result in the mobilization and spread of primary tumour cells to distant locations through the invasion of the basement membrane into the surrounding microenvironment (Shipitsin et al., 2007, Meyer et al., 2009, Creighton et al., 2010, Hayashida et al., 2011, Takebe et al., 2011). EMT is also linked to a dedifferentiation process, during which epithelial cells acquire stem-like properties (Mani et al., 2008, Morel et al., 2008, Lindley and Briegel, 2010, Li and Zhou, 2011).

Several studies have showed that the loss of E-cadherin is a hallmark of EMT (Figure 7) (Peinado et al., 2007, Kalluri and Weinberg, 2009, Li and Zhou, 2011, Takebe et al., 2011). E-cadherin is a cell-cell adhesion molecule that participates in homotypic, calcium-dependent interactions to form epithelial adherent junctions (Li and Zhou, 2011). Loss of E-cadherin expression has been associated with undifferentiated breast carcinomas and tumour progression both in human and canine mammary cancer (Knudsen and Wheelock, 2005, Gama et al., 2008b). β-cat is also an essential component of adherent junctions, since it provides the link between E-cadherin and the actin cytoskeleton, modulating cell-cell adhesion and cell migration.

Sequestration of β-cat in the cytoplasm is important for the preservation of epithelial features of cancer cells, and acquisition of the mesenchymal phenotype correlates with the movement of β-cat to the nucleus, where it becomes part of the TCF/LEF complexes (Kalluri and Weinberg, 2009). Recently, Li and Zhou (2011) showed that activation of β-cat pathway is required for the maintenance of EMT-associated stem cell-like properties (Li and Zhou, 2011).

Such as E-Cadherin, N-Cadherin is a transmembrane glycoprotein that promotes calcium-dependent cell-cell adhesive interactions, but it is expressed in neuronal tissue, muscle and fibroblasts. A growing body of evidence shows that most of highly invasive carcinoma cell lines that loose E-Cadherin, up-regulate N-Cadherin (Agiostratidou et al., 2007). Studies of Li and Zhou (2011) verified, by immunofluorescence and Western blotting, that N-Cadherin appears upregulated when breast cancer cells were transfected with Twist, a factor that induces the mesenchymal phenotype (Li and Zhou, 2011). Interestingly, the acquisition of a mesenchymal phenotype is associated with increased vimentin expression (Peinado et al., 2007, Meyer et al., 2009, Li and Zhou, 2011, Takebe et al., 2011). This mesenchymal marker is a type III intermediate filament protein, which appears upregulated in migratory cells. Vimentin expression, at sites of cell elongation, suggests its putative role in facilitating cell migration (Kokkinos et al., 2007).

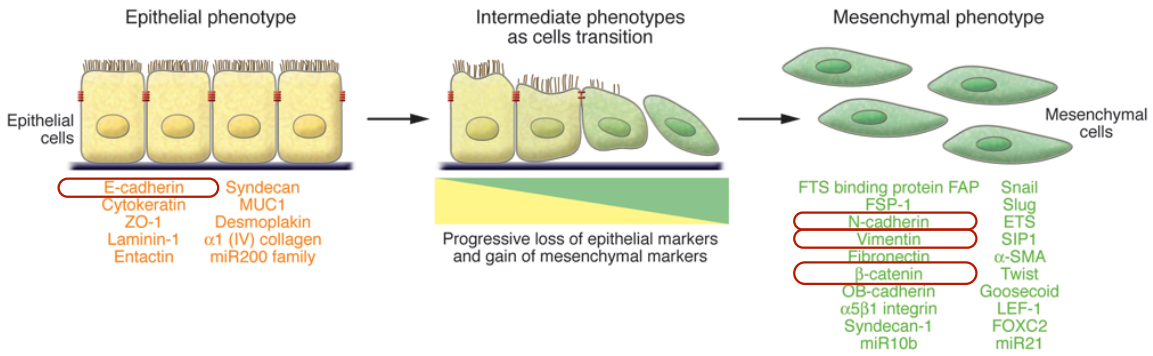


Figure 7. Epithelial-Mesenchymal Transition, a process characterized by loss of expression of markers of epithelial differentiation, acquisition of fibroblastic-like properties, reduced intercellular adhesion and increased motility (adapted from Kalluri and Weinberg, 2009).

4. CANINE MAMMARY CANCER AND CSCS

Currently, there are a small number of studies focused on the existence of CSCs in canine mammary cancer. Cocola et al. (2009) have generated non-adherent spheres from normal and neoplastic mammary tissue, which were enriched in early progenitor/stem cells and exhibited tumour-initiating potential (Cocola et al., 2009). Michishita et al. (2010) also characterized spheres derived from a canine mammary adenocarcinoma cell line, which showed a high expression of stem-cell related genes, confirming sphere culture system as a useful tool for the identification of CSCs in canine mammary cancer (Michishita et al., 2010). Another group showed that canine CSCs predominantly express mesenchymal markers and are more invasive than parental cells. Authors induced EMT by TGFβ treatment and observed that these cells have an increased ability to form tumourspheres, indicating that EMT induction can enrich for cells with CSC properties (Pang et al., 2011).

Interestingly, Ferletta et al. (2011) sorted a cell population with the ability to form spheroids, which were characterized by immunohistochemistry for several markers, such as CD44, CD49f (also known as α6-Int), CD24, Sox2 and Oct4 (Ferletta et al., 2011). Also Blacking et al. (2011) verified that CD44 expression is associated with proliferation in cultured canine cancer cells, suggesting that transient and fluctuating expression may limit its utility as a CSC marker (Blacking et al., 2011) in canine mammary cancer. It was also showed that a population of cells with high ALDH activity were enriched in a $CD44^{+}CD24^{-}$ phenotype, characterized by self-renewal capacity and enhanced tumourigenicity in immunodeficient mice, concluding that ALDH activity could be a good marker to isolate and identify CSCs in canine mammary carcinoma cell lines (Michishita et al., 2012).

Recently, Pang et al. (2011) showed that canine CSCs are relatively resistant to cytotoxic effects of common chemotherapeutic drugs and ionizing radiation, suggesting that failure of clinical therapy to eradicate canine mammary cancer may be due to the survival of CSCs (Pang et al., 2011). Treatment options are still rather limited and surgery remains the treatment of choice for the majority of dogs with mammary gland tumours (Rutteman et al., 2001). If stem/progenitor cells are really the targets for transforming events in canine mammary gland, as seems to be the case in human breast cancer (Charafe-Jauffret et al., 2008), the elucidation of the molecular pathways regulating self-renewal activity of CSCs and their interaction with the microenvironmental niche, will provide potential therapeutic targets for this disease.

AIMS

The main goal of this work was to characterize stem cell properties in canine mammary cancer. With this purpose, we defined the following specific aims:

1. To characterise the phenotype and function of canine mammary carcinoma cell lines, which have been previously established by our group.
2. To characterize the stem cell-related phenotype of these cells, by analysing the expression of human breast CSC markers such as ALDH1, CD44 and CD24.
3. To investigate the ability of these cells to grow in anchorage independent conditions as spheroids.
4. To investigate a possible link between EMT and CSC phenotype/properties, by analysing the expression of EMT markers in these canine cell lines.
5. To investigate the prevalence of human CSC markers in a series of canine mammary carcinomas, by analysing ALDH1, CD44 and CD24 expression by immunohistochemistry and correlating the results with some available clinicopathological data.

MATERIAL AND METHODS

CANINE MAMMARY TUMOUR CELL LINES AND CELL CULTURE

Three canine mammary tumour cell lines (CMT-1m, CMT-2p and CMT-3p) were previously established by our group. The first cell line is derived from a metastatic lesion (CMT-1m) and the two others from primary carcinomas (CMT-2p and CMT-3p), all obtained from canine patients bearing spontaneous mammary tumours.

The three cell lines were cultured in RPMI 1640 with 25mM Hepes and L-Glutamine (Lonza, Basel, Switzerland), supplemented with 12% fetal-inactivated bovine serum (FBS, Lonza) and with 1% antibiotic solution (Penicillin/Streptomycin, Invitrogen, Carlsbad, CA). All cell lines were routinely cultured in a humidified atmosphere with 5% CO_2 and at 37^{o}C. Cells were used in experiments when 70-80% of confluence was reached.

TISSUE SAMPLES, CLINICOPATHOLOGICAL AND FOLLOW-UP DATA

Canine mammary tumours were obtained from the archives of the Histopathology Laboratory of the University of Trás-os-Montes and Alto Douro, Vila Real. Tumour samples were surgically removed from 112 female dogs in private clinical practices (the majority from the Northern region of Portugal) or in the hospital of the above-mentioned institution. The material had been fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (3µm) were cut and stained with haematoxilin and eosin (HE) for histological examination and immunohistochemistry stainings.

Clinicopathological parameters collected from each case included age, tumour size, tumour histological type and grade, ulceration, lymphovascular invasion and lymph node involvement. Tumour size was defined as the maximum diameter and tumours were grouped according to the TNM World Health Organization (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 two observers from haematoxylin and eosin (HE) stained sections, according to the WHO criteria for canine mammary neoplasms (Misdorp *et al*., 1999). Histological grade was evaluated 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.

Follow-up information was available for ninety-five tumour cases, ranging from a minimum of one to a maximum of 24 months after the diagnosis, with a median overall survival time of 20 months. 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 did not 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.

WESTERN BLOT

Cells were lysed with PBS containing 1% Nonidet-P40 (Sigma, Germany), 1% Triton X100 (Sigma, Germany) and 1:7 Protease Inhibitors Cocktail (Roche Diagnostics Gmbh, Mannheim, Germany). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Richmond, CA) and equal amounts were resolved on an 8% denaturating polyacrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, UK) at 100V for 90min. After blocking nonspecific binding with 5% nonfat dry milk in PBS containing 0.5% Tween 20, each membrane was incubated at room temperature with each of the antibodies showed in Table 1, with α-tubulin or β-actin antibodies used as loading controls. After washing four times with 5% milk buffer for 5 minutes, membranes were incubated with the secondary antibodies (1:2000, Santa Cruz Biotechnology Inc., Heidelberg, Germany) for 45 minutes and washed six times for 5 minutes with PBS containing 0.5% Tween 20. Detection was assessed using the ECL chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Table 1 – Antibodies and conditions used for Western Blot.

Antibody	Clone	Manufacturer	Blocking buffer	Primary antibody		Secondary antibody
				Dilution	Incubation	
α-tub	DM1A	Sigma, Germany	Milk buffer 5%	1:1000	1 hour	Mouse
β- act	I19	Santa Cruz, USA	Milk buffer 5%	1:1000	1 hour	Goat
PgR	SP2	Novocastra, UK	Milk buffer 5%	1:50	1 hour	Mouse
ER	6F11	Novocastra, UK	Milk buffer 5%	1:50	1 hour	Mouse
p63	4A4	NeoMarkers, USA	Milk buffer 5%	1:200	1 hour	Human
E-Cad	24E10	Cell Signaling, USA	Milk buffer 5%	1:1000	1 hour	Rabbit
P-Cad	56	BD Transduction, USA	Milk buffer 5%	1:500	1 hour	Mouse
β-cat	14	BD Transduction, USA	Milk buffer 5%	1:1000	1 hour	Mouse
CD44v6	VFF-7	Abcam, UK	Milk buffer 5%	1:500	1 hour	Mouse
CD44s	IM7	Santa Cruz, USA	Milk buffer 5%	1:250	1 hour	Rat
ALDH1	EP1933Y	Abcam, USA	Milk buffer 5%	1:1000	Overnight	Rabbit
β4-Int	SC9090	Santa Cruz, USA	Milk buffer 5%	1:2000	1 hour	Rabbit
α6-Int	4C1	Sigma, Germany	Milk buffer 5%	1:1000	1 hour	Rabbit
Fib	2755-8	Santa Cruz, USA	Milk buffer 5%	1:1000	1 hour	Mouse
N-Cad	32	BD Transduction, USA	Milk buffer 5%	1:500	1 hour	Mouse
Vim	V9	DAKO, USA	Milk buffer 5%	1:1500	1 hour	Mouse

IMMUNOFLUORESCENCE

Cells were cultured on glass coverslips, and fixed with 4% paraformaldehyde (during 20 minutes). After fixation, cells were treated with 50mM NH_4Cl for 10 minutes, washed with PBS, and permeablilized with 0.1% Triton X-100 in PBS for 5 minutes, at room temperature. Non-specific binding was blocked with PBS containing 5% BSA, for 30 minutes, at room temperature. Cells were then stained with a specific primary antibody. The antibodies and conditions used are listed in Table 2. In the majority of cases, primary antibody detection was performed using a secondary antibody, at a 1:1000 dilution. After washing with PBS, each sample was mounted with Vectashield Mounting Medium with 4,6-diamidine-2-phenylindolendihydrochrolide (DAPI) (Vector Laboratories, California, USA). The staining was observed with a Zeiss microscope (Imager Z1), and images were acquired using the Axiovision software.

Table 2 – Antibodies and conditions used for Immunofluorescence.

Antibody	Clone	Manufacturer	Primary antibody		Secondary antibody
			Dilution	Incubation	
CD44s	IM7	Santa Cruz, USA	1:50	Overnight	Rat
CD44v6	VFF-7	Abcam, USA	1:50	Overnight	Mouse
CD24	M1/69	BD Biosciences, USA	1:10	Overnight	- (PE)
ALDH1	EP1933	Abcam, UK	1:500	Overnight	Rabbit
α6-Int	GoH3	BD transduction, USA	1:10	1:30h	- (FITC)
β4-Int	439-9B	BD transduction, USA	1:10	1:30h	- (PE)
P-Cad	C13F9	Cell Signalling, USA	1:50	Overnight	Rabbit
E-Cad	24E10	Cell Signalling, USA	1:100	Overnight	Rabbit
β-cat	14	BD transduction, USA	1:100	Overnight	Mouse

SLOW AGGREGATION ASSAY

This assay was used to evaluate cell-cell adhesion capacity. For slow aggregation assay in semi-solid substratum, $2x10^4$ cells of each cell line were seeded per well on a solidified agar in a 96 well plate. Semi-solid medium was prepared with Bacto-agar in PBS. After boiling, it was poured immediately into the plates, in order to cover wells with an agar layer to prevent cell-substratum adhesion. After trypsinization and cell counting, cells were seeded (six wells per cell line). and plates were incubated at 37°C in a humified atmosphere with 5% CO_2 in air. The aggregate formation was evaluated with an inverted microscope after 24, 48, and 72 hours. The MCF7.PCad and MDA-MB-231 cell lines were included as positive and negative controls, respectively.

BROMODEOXYURIDINE (BrdU) INCORPORATION PROLIFERATION ASSAY

In BrdU procedure, cells were cultured on glass coverslips to a confluence of 50%. After 24h, 10µL of BrdU/mL was added to each well for 1h. The medium containing BrdU was rejected and after two washing steps with sterile PBS, cells were fixed with 4% paraformaldehyde in PBS for 30min. Cells were then treated with 2M HCl for 20 minutes and washed with PBS at room temperature. Non-specific binding was blocked washing two times with PBS-Tween20 0.5% containing 0.05% BSA, for 10 minutes, at room temperature. Subsequently, cells were stained with a specific primary antibody anti-BrdU (Clone Bu20a, mouse, DAKO, USA) at a dilution of 1:10 in PBS-Tween20 0.5%-BSA 0.05% for 1 hour at room temperature. Following additional washes with PBS-Tween20 0.5%-BSA 0.05%, the detection of the primary antibody was performed using

the secondary antibody, Alexa Fluor 488 (Invitrogen, Carlsbad, CA) at a dilution of 1:500 in PBS-Tween20 0.5%-BSA 0.05% at a room temperature for 30min, in the dark. After washing with PBS-Tween20 0.5%-BSA 0.05%, each sample was mounted with Vectashield Mounting Medium with 4,6-diamidine-2-phenylindolendihydrochrolide (DAPI) (Vector Laboratories, California, USA). The staining was observed with a Zeiss microscope (Imager Z1), and images were acquired using the Axiovision software.

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE dUTP NICK END LABELING (TUNEL) CELL DEATH ASSAY

Cells were grown in a serum starved medium that was used as an apoptotic stimulus; the attached and floating cells were pooled, pelleted by centrifugation, washed in PBS, and fixed with 4% formaldehyde for 30 minutes. Cytospins preparations were used for TUNEL assay, which was carried out according to manufacturer's recommendations (TUNEL kit, Roche Diagnostics Gmbh, Mannheim, Germany). Approximately 500 apoptotic and non-apoptotic cells were counted per slide. Death index was calculated as the percentage of death cells over the total counted cells.

MATRIGEL INVASION ASSAY

Matrigel invasion assay was performed using 8µm pore size BD BioCoat™ Matrigel Invasion Chambers (BD Biosiences, NJ, USA). In the upper compartment of the chamber, $5x10^4$ cells of each cell line were added, whereas in the lower compartment, only fresh medium supplemented with 12% FBS was present. After 24h of incubation at 37°C, the upper surface of the filter was cleared from non-invasive cells with a cotton swab and washed with PBS. The remaining (invasive) cells, which were attached to the lower surface of the filter, were fixed with cold methanol and mounted with Vectashield (Vector Laboratories, Inc, Burlingame, CA) containing DAPI. Invasive cells were scored by counting the cells in the filter with a fluorescence microscope (Leica DM 2000), at 200X of magnification. The invasive MDA-MB-231 cell line was routinely included as a positive control and MCF7 cell line as a negative control.

GELATIN AND β-CASEIN ZYMOGRAPHY

The conditioned medium collected from the several cell cultures, which were grown in 6-well plates coated with collagen type I, was analysed for proteinases activity using gelatin and β-casein zymography. Gelatin and β-casein gels were loaded with 12 µg of protein per sample.

Samples were mixed with sample buffer [0.03% bromophenol blue, 0.25M Tris-HCl pH 6.8, 10% SDS (w/v) and 4% sucrose (w/v)] and electrophoresed, under non-reducing conditions, on 10% polyacrilamide gels containing 0.1% (w/v) gelatin or β-casein from bovine milk (Sigma, Germany). After electrophoresis, gels were washed twice, for 30 minutes, in 2% (v/v) Triton X-100 (Sigma, Germany) at room temperature, in order to remove SDS. Then, they were incubated in a Substrate Reaction Buffer for 20h in case of the gelatin gels [50 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.5] or 72h for β-casein gels [0.2M NaCl, 5 mM CaCl2, 1% (v/v) Triton X-100 in 50mM Tris-HCl, pH 7.4], and finally stained with Coomassie Blue Staining Solution [0.1% (w/v) Coomassie Blue R250 in 10% (v/v) acetic acid and 40% (v/v) methanol], for 25 minutes. The gels destaining was performed in a solution with 20% methanol and 10% acetic acid, until bands start to become visible. Enzymatic activity was visualized as a clear band against the blue background of stained gels, and MMPs were identified by their molecular weight. Quantification of band density was carried out using the Quantity One software (version 4.0, BioRad, Hercules, CA).

FLOW CYTOMETRY

Cells were washed twice with sterile PBS and then harvested with versene/0.48mM EDTA (Invitrogen, Carlsbad, CA). Detached cells were washed with PBS supplemented with 0.5% FBS (stain buffer), centrifuged 1200 rpm for 5 min at room temperature and re-suspended in stain buffer. This solution was passed through a 25G needle, using a syringe, to separate cells into a single cell suspension. Then, cells were incubated with phycoerytrin (PE)-conjugated anti-CD24 (clone M1/69, BD Biosciences, San Diego) and anti-CD44 (clone IM7, Santa Cruz, USA). Primary antibodies were added to the cell suspension and incubated at 4°C in the dark for 30 min. A secondary antibody conjugated with fluorochrome Alexa 488 was incubated afterwards, at the same conditions used for primary antibodies. A cell viability marker was included (violet fluorescent reactive dye, Invitrogen, Carlsbad, CA), in order to remove dead cells. The labelled cells were washed in the stain buffer and then analysed on a FACS Canto II (BD Biosciences). All antibodies were used at a concentration of 1:100 in stain buffer.

ALDEFLUOR ASSAY

The ALDEFLUOR kit (Stem Cell Technologies, Grenoble, France) was used to isolate the population with high ALDH enzymatic activity, using a FACS Canto II (BD Biosciences), according to the manufacturer instructions. Briefly, cells were incubated in ALDEFLUOR assay buffer containing

ALDH substrate, Bodipy®-aminoacetaldehyde (BAAA) (1 µmol/L per 1 x 10^6 cells). In each experiment, a sample of cells was stained, under identical conditions, with 50 mmol/L of diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, as a negative control. A cell viability marker was included (violet fluorescent reactive dye, Invitrogen, Carlsbad, CA), in order to remove dead cells. Cells were washed in assay buffer and left at 4°C, until measurements and analyses had been performed.

MAMMOSPHERE ASSAY

Cell lines were trypsinized, neutralized with complete medium, centrifuged 1200 rpm for 5 min at room temperature and re-suspended in cold PBS. This solution was passed three times through a 25-G needle, using a syringe, to separate cells into a single cell suspension. Cells were plated at 500 cells/cm^2 in low attachment plates, containing mammosphere culture medium [DMEM:F12 medium, without phenol red, and supplemented with B27 (which is a serum that excludes constituents that lead to cell differentiation, Invitrogen, Carlsbad, CA; diluted 1:50)], hydrocortisone 500 ng/ml (Sigma, Germany), insulin 40 mg/ml (Sigma, Germany) and recombinant human EGF 20 ng/ml (Sigma, Germany).

Mammospheres of at least 50 µm in diameter (determined by using an eyepiece graticule with crossed scales) were counted on day 6 after plating. Mammosphere forming efficiency (MFE) was calculated by dividing the number of mammospheres (≥50 µm) formed by the original number of single cells seeded, being expressed as a percentage.

IMMUNOHISTOCHEMISTRY

This technique was used to investigate the expression and subcellular localization of human breast CSC markers (CD44, CD24 and ALDH1) in a series of 112 canine mammary carcinomas. The antigen retrieval conditions, antibodies, dilutions and suppliers are listed in Table 3. Antigen retrieval was performed in a 10 mM citrate buffer, pH=6.0 (3 x 5 min) in a 750W microwave. After cooling 20 minutes at room temperature, tissue sections were immersed in 3% hydrogen peroxide (H_2O_2) (Panreac, Spain) and distilled water during 30 minutes to block endogenous peroxidase activity. Non-specific staining was eliminated by incubation in a blocking serum (Ultra V Block, LabVision, USA) for 5 minutes. Excess serum was removed and the slides were incubated in a humid chamber with the respective primary antibodies. Primary antibody incubation was performed for 1 hour at room temperature or overnight at 4°C. After incubation,

the slides were washed and incubated with secondary antibody associated with HRP-labelled (horseradish peroxidase) polymer for 30 minutes (for CD44) or incubated with biotinylated goat anti-polyvalent (Labvision, USA), followed by streptavidin-conjugated peroxidase (Labvision, USA) during 10 minutes (for CD24 and ALDH1). Sections were rinsed thoroughly with PBS between each step of the procedure. Subsequently, the staining was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) chromogen with H_2O_2 in PBS buffer for 10 minutes. Tissues were then counterstained with Gill's haematoxylin, dehydrated and cover-slipped using a permanent mounting solution (Entellan, Merck). Paraffin sections of liver (for ALDH1) and normal mammary tissue (for CD24 and CD44) were included as positive controls, as well as to ensure consistency between consecutive runs. Negative controls were carried out by replacing the primary antibody with PBS.

Table 3 – Antibodies and conditions used for Immunohistochemistry.

Antibody	Clone	Manufacturer	Antigen retrieval	Primary antibody		Detection system
				Dilution	Incubation	
CD44	IM7	Santa Cruz, USA	Citrate buffer	1:400	Overnight	Streptavidin-biotin-peroxidase
CD24	Ab2-SN3b	Neomarkers, USA	Citrate buffer	1:100	Overnight	HRP-labelled polymer
ALDH1	EP1933Y	Abcam, UK	Citrate buffer	1:100	Overnight	HRP-labelled polymer

Two pathologists performed the evaluation of the immunohistochemical results. The expression of all proteins were evaluated as described for human breast cancer (Ricardo et al., 2011). Briefly, CD44 and CD24 staining were detected at the membrane of tumour cells and the scoring was considered as follows: 0, <10% of positive tumour cells; 1+, 10-25% of positive tumour cells; 2+, 25-50% of positive tumour cells; 3+, more than 50% of positive tumour cells. Cytoplasmic staining was also considered for CD24. For CD44, cases classified as 0 were considered negative, whereas 1+, 2+ and 3+ were established as positive cases. For CD24, the cases were divided into negative/low (-/low), when considered 0 or 1+, or in positive cases, when classified as 2+ or 3+. Immunohistochemical staining of ALDH1 was classified as positive when more than 1% of tumour cells showed clear cytoplasmic staining, as previously described (Ginestier et al., 2007, Deng et al., 2010, Ricardo et al., 2011). Stromal expression of ALDH1 was also classified in two categories: none/weak, or moderate/strong, as previously described by Resetkova et al. (2010) (Resetkova et al., 2010).

The series had previously been partially studied by immunohistochemistry with respect to Ki-67 index (MIB-1 clone, 1:50, Dakocytomation), which was calculated as the percentage of tumour cells that exhibited positive nuclear staining for Ki-67.

STATISTICAL ANALYSIS

Mammospheres forming ability (size and number) was compared using two-tailed unpaired t-test. Flow cytometry data was analysed with the Flowjo software package (TreeStar, Ashland, OR, USA).

Associations between CSC markers expression and categorical variables were performed by using the chi-square test or Fisher's exact test (two-sided). Associations between CSC markers expression and Ki-67 index (continuous variable) were assessed by the non-parametric Mann-Whitney test. In order to determine the effect of CSC markers on prognosis, survival curves were generated by the Kaplan-Meier method and the survival rates compared using the log-rank test. These statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, Chicago, USA), 11.5 version. A p value<0.05 was considered statistically significant.

RESULTS

1. CHARACTERISATION OF THE PHENOTYPE AND FUNCTION OF THE CANINE MAMMARY TUMOUR CELL LINES CMT-1m, CMT-2p AND CMT-3p

In order to better interpret the results related with cancer stem-like properties observed with the three canine mammary tumour cell lines CMT-1m, CMT-2p and CMT-3p used in this study (and previously established by our group), we first decided to characterise them concerning the expression of some molecular markers and functional activities.

The selected markers for analysis were breast cancer-related receptors, such as ER, PgR, and HER2, as well as the myoepithelial marker p63, the mesenchymal marker vimentin, and the adhesion molecules E-cadherin (E-Cad) and P-cadherin (P-Cad). The expression of these markers was assessed by western blot analysis. Interestingly, differential protein banding patterns were observed in the three cell lines when stained with Ponceau, indicating clear differences between the three distinct cell lines.

As shown in Figure 8A, a positive expression for ER was found in all the cell lines, although with lower levels in CMT-3p. The positive expression of E-Cad was similar amongst all the cell lines. The other markers, such as PgR, HER2, p63, vimentin, and P-Cad were not detected in any of the three cell lines.

The cellular localization of cadherins was also evaluated by immunofluorescence (Figure 8B), and we could confirm the result obtained by western blot: P-Cad was absent in all the three cell lines, whereas E-Cad expression was found in all cell lines; however, while E-Cad was mainly normally localized at the membrane cell-cell contacts in CMT-1m and CMT-3p cells, it was abnormally localized in CMT-2p, showing a cytoplasmatic expression.

To evaluate the functionality of the adherens junctions in the ability to form cell-cell aggregates, we performed a classic cell-cell aggregation assay. After 24 hours, CMT-1m and CMT-3p showed prominent aggregates, as can be seen in Figure 9; however, these showed different patterns, since CMT-1m formed compact aggregates and CMT-3p formed irregular aggregates, with high levels of proliferation. CMT-2p cells did not aggregate, being very similar to the negative control, even after 72 hours.

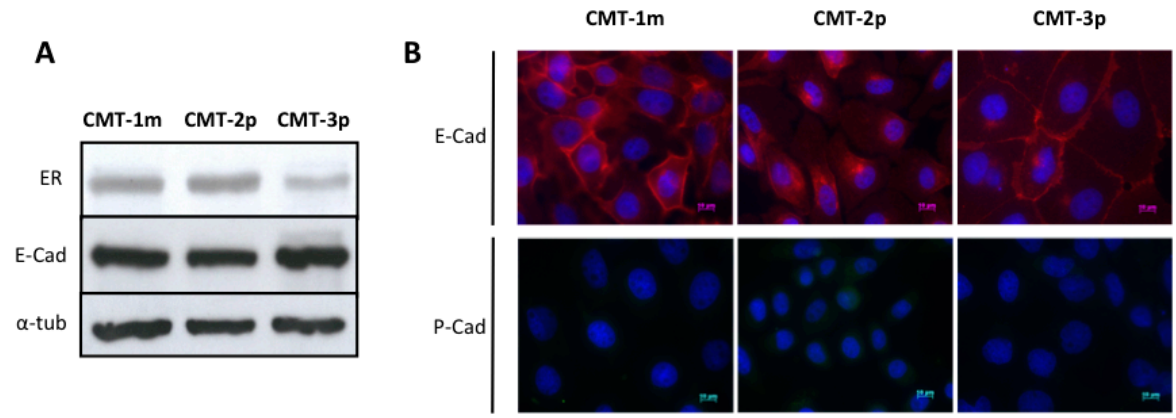


Figure 8. **(A)** Western blot showing expression of the molecular markers ER and E-Cad in CMT-1m, CMT-2p and CMT-3p cell lines. ER and E-Cad were detected in all three cell lines, although ER expression was lower in CMT-3p. **(B)** Immunofluorescence to evaluate the cellular localization of the adhesion molecules E-Cad and P-Cad. P-Cad was not detected in none of the three cell lines; E-Cad was located at the cell membrane in CMT-1m and CMT-3p cells and abnormally expressed in CMT-2p, showing a cytoplasmic expression. Human breast cancer cell lines, MCF7 and SKBr3 (not shown), were used as positive and negative controls, respectively. Scale bar=10µm

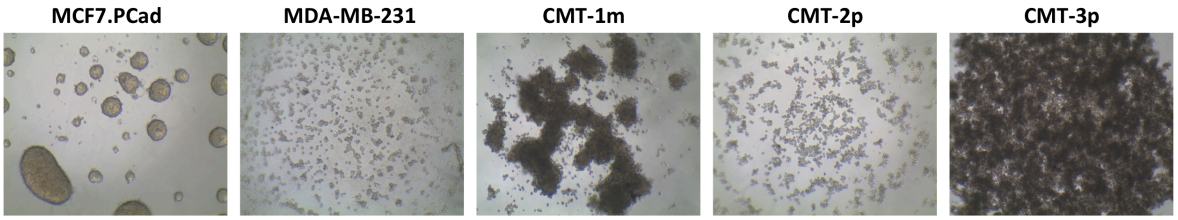


Figure 9. Cell aggregation performed by the slow cell aggregation assay protocol, where the human breast cancer cell lines MCF7.PCad and MDA-MB-231 were used as positive and negative controls, respectively. These images show aggregates formed after 24h. CMT-1m showed compact aggregates, CMT-2p was similar to the negative control, showing no aggregation, and CMT-3p cells showed aggregation without compaction. Original magnification: 40x.

The proliferation and cell death rates of the three cell lines were also evaluated. CMT-3p cells are more proliferative, but also the ones with higher levels of cell death, as seen in Figure 10**.**

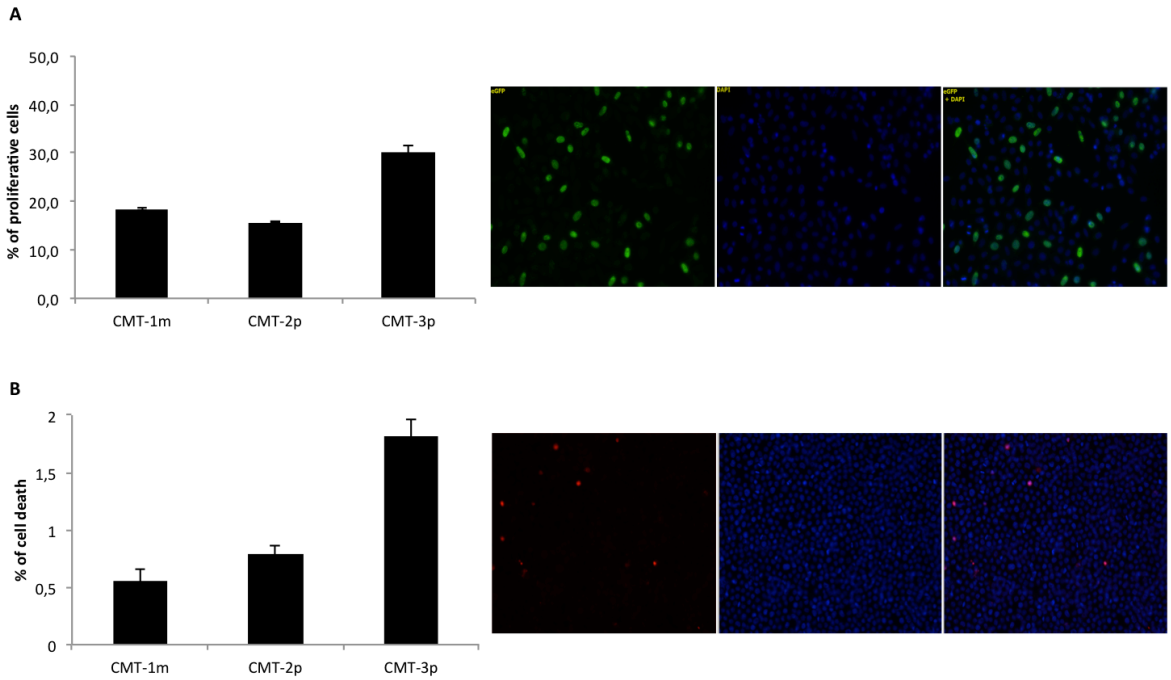


Figure 10. Graphical representations of the percentage of **(A)** proliferative cells and **(B)** percentage of cell death in CMT-1m, CMT-2p and CMT-3p canine mammary tumour cell lines. On the right, representative images can be seen for the CMT-2p cell line. Each bar represents the mean number ± SEM of three independent experiments. Original magnification: 200x.

Additionally, to assess the invasion capacity of each cell line, we performed *in vitro* invasion assays using Matrigel-coated transwell chambers. As shown in Figure 11**,** we can observe that CMT-2p cells are not invasive, since there were almost no cells with invasive capacity. Only an average of 0.17% of CMT-2p cells were able to invade, followed by CMT-1m, with an average of 2% of invasive cells, and finally CMT-3p, with almost 10% of invasive cells.

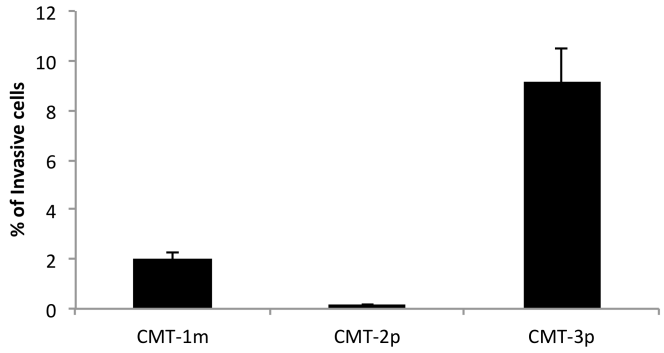


Figure 11. Invasion ability through an artificial extracellular matrix (Matrigel) assessed by Transwell assays. CMT-2p cells are non invasive, whereas CMT-3p cells are the ones presenting a high invasion capacity. Each bar represents mean number ± SEM of invaded cells after 24 hours (three independent experiments).

Finally, since MMPs are a family of proteases which are well known to be involved in cell invasion induction, namely in the ECM degradation components, their activity levels were also assessed in the conditioned medium from the three canine mammary tumour cell lines, using β-casein [to assess MMP-2 (66 KDa), MMP-1 (42 KDa) and MMP-7 (28 KDa) activity] and gelatin [to assess MMP-2 (66 KDa) and MMP-9 (92 KDa activity)] zymography. As shown in Figure 12, CMT-1m cells showed MMP-2, MMP-1, and MMP-9 protease activity. However, CMT-2p cell lines showed a higher activity of MMP-2 and MMP-1, whereas CMT-3p cells showed active levels of MMP-7 and MMP-9.

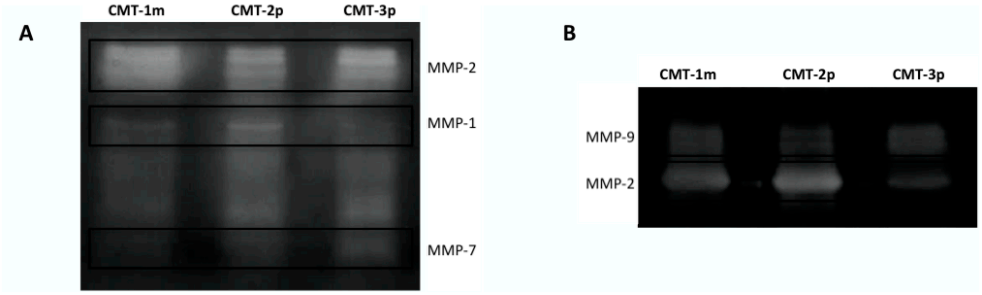


Figure 12. (A) β-casein zymography, which showed MMP-2 activity in the three cell lines. Concerning MMP-1, CMT-1m cells showed low activity, whereas CMT-2p cells showed increased levels. MMP-7 activity was only found in CMT-3p cells. **(B)** Gelatin zymography revealed MMP-9 activity in CMT-1m and CMT-3p cell lines. Concerning MMP-2 activity, the results confirmed the ones obtained with b-casein zymography, showing that CMT-2p cells are the ones with higher activity levels of this protease.

2. INVESTIGATING THE CANCER STEM CELL PHENOTYPE OF THE CANINE MAMMARY TUMOUR CELL LINES CMT-1m, CMT-2p AND CMT-3p

To address if CMT-1m, CMT-2p and CMT-3p cell lines could be distinguished phenotypically by the expression of human breast CSC markers, the expression of α6 and β4 integrins, CD44v6, CD44s, CD24 and ALDH1 (ALDH1A1 isoform) was evaluated by western blot and immunofluorescence, as shown in Figure 13.

Analysing the results, it is interesting to note that the canine mammary tumour cell line that expresses more CSC markers is the CMT-2p, since it highly expresses α6 and β4 integrins, CD44v6 and ALDH1; CMT-1m cells also express these same markers, but in a weakly way compared with CMT-2p, but strongly expresses CD44s. Regarding CMT-3p cell line, this seems to be the one expressing less CSC markers, since it only expresses CD44s, but low CD44v6 and β4-Int, being completely negative for α6-Int and ALDH1. None of the cell lines express membrane CD24, as observed by immunofluorescence.

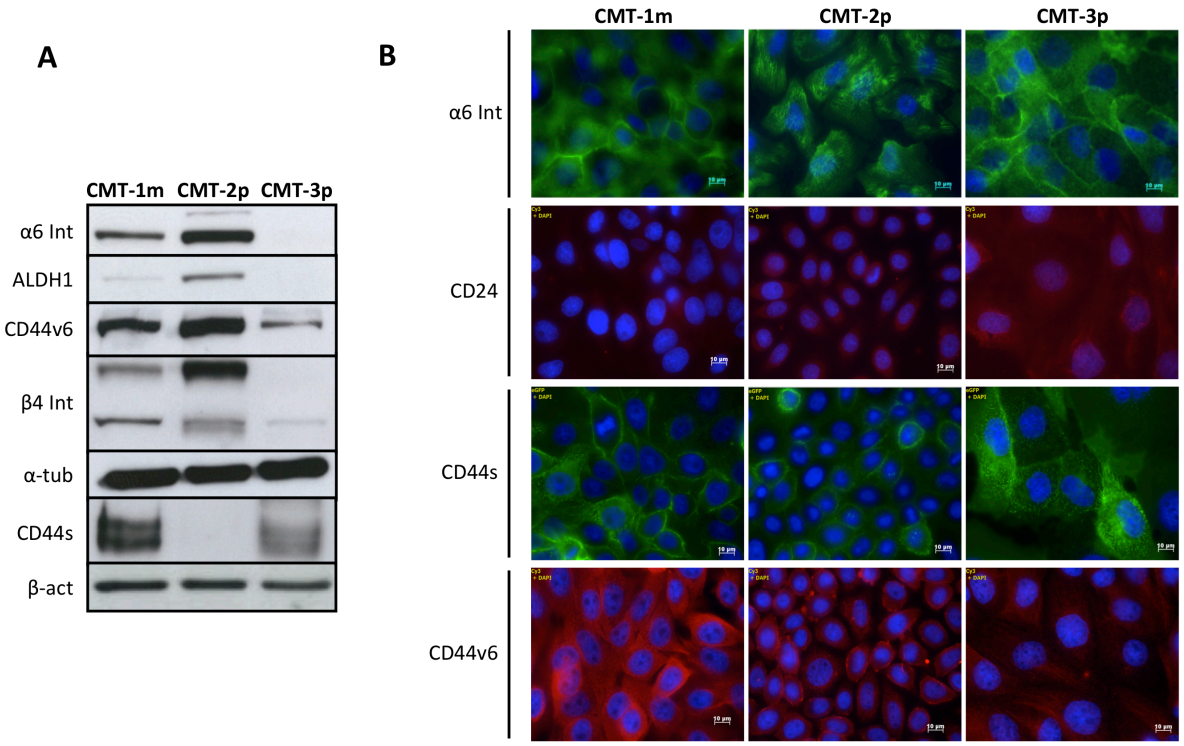


Figure 13. (A) Western blot showing expression of some breast CSC markers in CMT-1m, CMT-2p and CMT-3p cell lines, namely α6 and β4 integrins, ALDH1, CD44v6 and CD44s. **(B)** Immunofluorescence to confirm the expression and localization of some breast CSC markers, namely a6 integrin, CD24, CD44s and CD44v6. Original magnification: 630x. Scale bar=10μm.

We subsequently asked whether any of the three cell lines was enriched for the most commonly used human breast CSC phenotype, the $CD44^{+}CD24^{-/low}$ phenotype defined by Al-Hajj and colleagues (Al-Hajj et al., 2003). Therefore, the combined expression of CD44/CD24 was determined by flow cytometry in the three canine mammary tumour cell lines. Results are summarized in Figure 14. In fact, flow cytometry analysis allowed us to divide the populations according to different levels of CD24 and CD44 expression.

Although not detected by immunofluorescence, CD24 was differentially detected amongst the cell lines analysed by flow cytometry, being its expression higher in CMT-1m cell line and lower in CMT-2p and CMT-3p. The expression of CD44 was similar, being high in all cell lines. Therefore, we observed that CMT-2p and CMT-3p was enriched for the most commonly used CSC phenotype, the $CD44^{+}CD24^{-/low}$ and CMT-1m are mainly constituted by a $CD44^{+}CD24^{+}$ cell population.

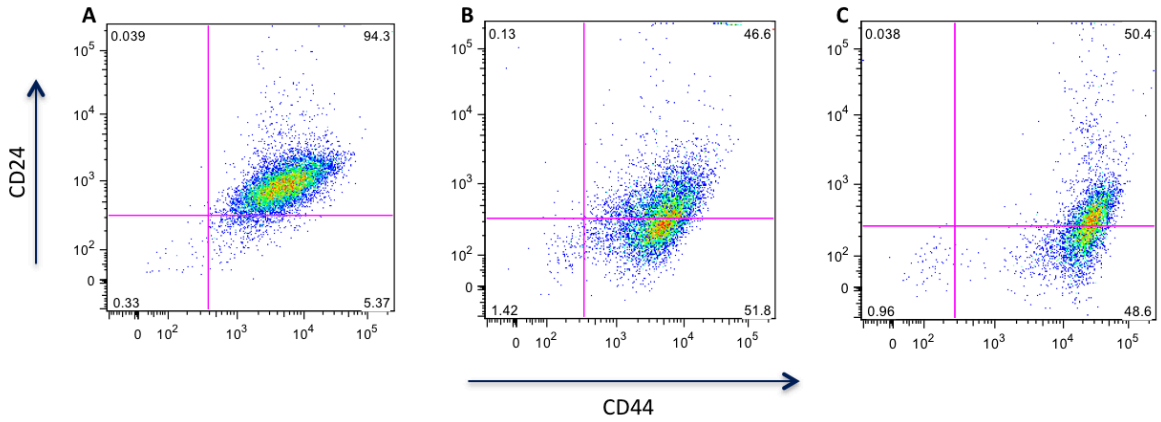
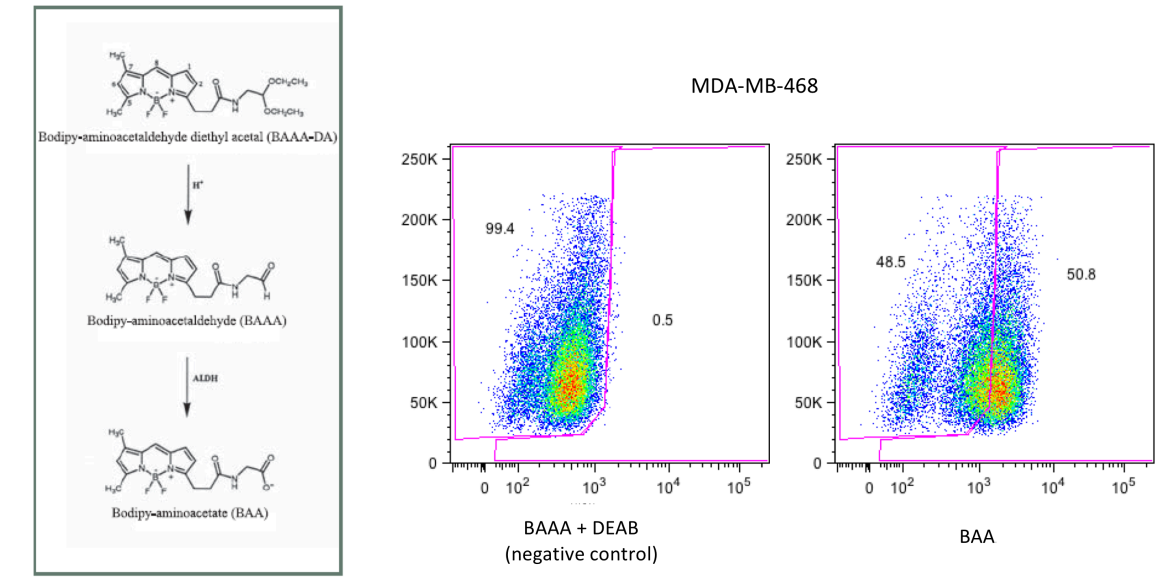


Figure 14. Identification of the subpopulations defined by CD44-FITC and CD24-PE expression in canine mammary tumour cell lines by flow cytometry.

In this study, we still used the ALDEFLUOR assay to analyse the canine mammary tumour cell lines, which is a useful tool for detecting the enzymatic activity of the ALDH isoforms, such as ALDH1A1 and ALDH1A3 and the principle of this assay is demonstrated in Figure 15. A summary of the percentage of the putative stem cell fraction obtained by the ALDEFLUOR assay is presented in Figure 16. As the results showed, the three cell lines have ALDH activity and no significant differences were found amongst the cell lines.



In: ALDEFLUOR ® technical bulletin (StemCell Technologies)

Figure 15. ALDEFLUOR assay principle and gating strategy to identify the ALDEFLUOR positive subpopulation in canine mammary tumour cell lines, where a human breast cancer cell line (MDA-MB-468) was used as a positive control. BAAA – ALDH1 substrate, DEAB – ALDH1 inhibitor.

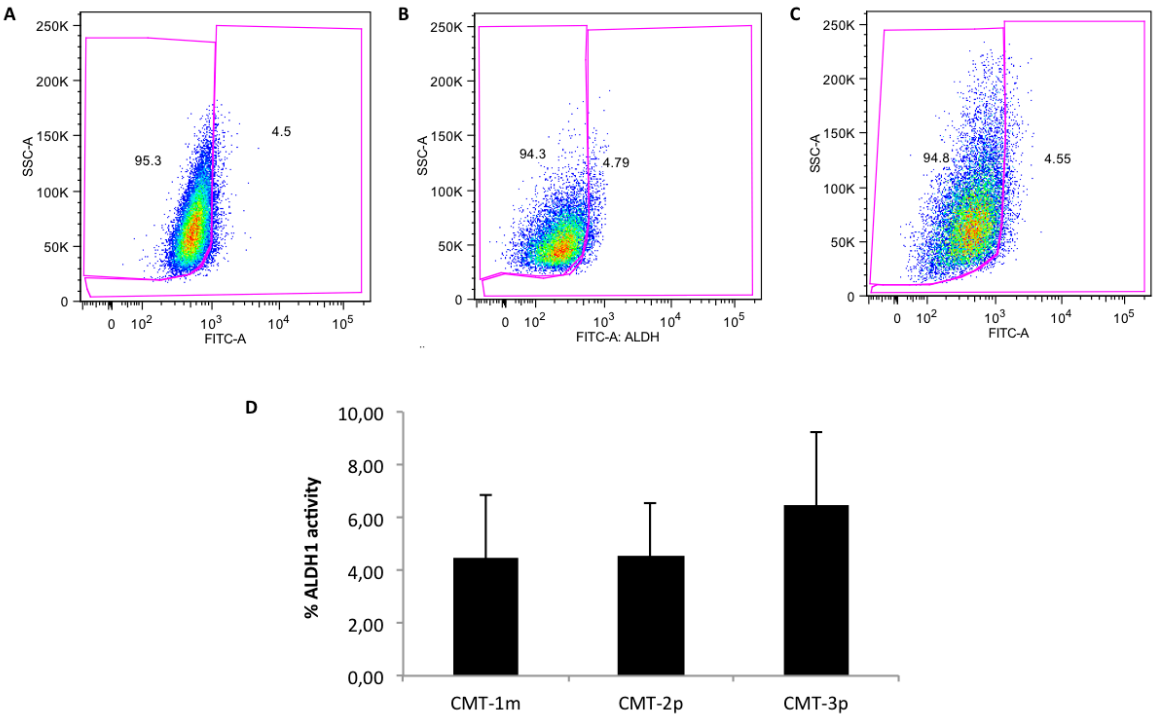


Figure 16. (A) (B) (C) Images exemplifying the results of ALDEFLUOR assay in CMT-1m, CMT-2p and CMT-3p, respectively. **(D)** Percentage of the ALDEFLUOR positive subpopulation defined by the ALDEFLUOR assay (StemCell Technologies) in the three canine mammary tumour cell lines. Each bar represents mean number ± SEM of the three independent experiments.

3. STUDYING THE ABILITY OF THE CMT-1m, CMT-2p AND CMT-3p CANINE MAMMARY TUMOUR CELL LINES TO GROW IN ANCHORAGE INDEPENDENT CONDITIONS AS SPHEROIDS

Dontu et al. (2003) showed that ALDEFLUOR-positive cells were able to form spheres through a specific assay and Ginestier et al. (2007) found that mammospheres were enriched for ALDH1. The referred assay has been used to isolate cells that seem to be breast cells with stem cell properties, since this mammary epithelial cells could survive and proliferate in anchorage-independent conditions (Dontu et al., 2003), showing self-renewal capacity.

Thus, this assay has been applied to the three canine mammary tumour cell lines, which were able to grow in anchorage independent conditions, forming multicellular structures that can encompass different morphologies. An example of the mammospheres obtained is depicted in Figure 17A**.** Our microscopical analysis showed that mammospheres could be roughly divided into two main groups: a compact spherical structure (as showed by CMT-1m and CMT-3p cells), containing inside the stem/progenitor cells. This central sphere can be surrounded by differentiated cells that either resisted death by anoikis or are derived from the central structure. On the other hand, some mammospheres have a loose grape-like structure and no central

compact aggregate can be distinguished (as showed by CMT-2p). When mammosphere forming efficiency (MFE) was determined (Figure 17B), we could observe that stem/progenitor cells in breast cell lines represent a small fraction of the whole population, with the MFE ranging from 1.07 to 3.05%.

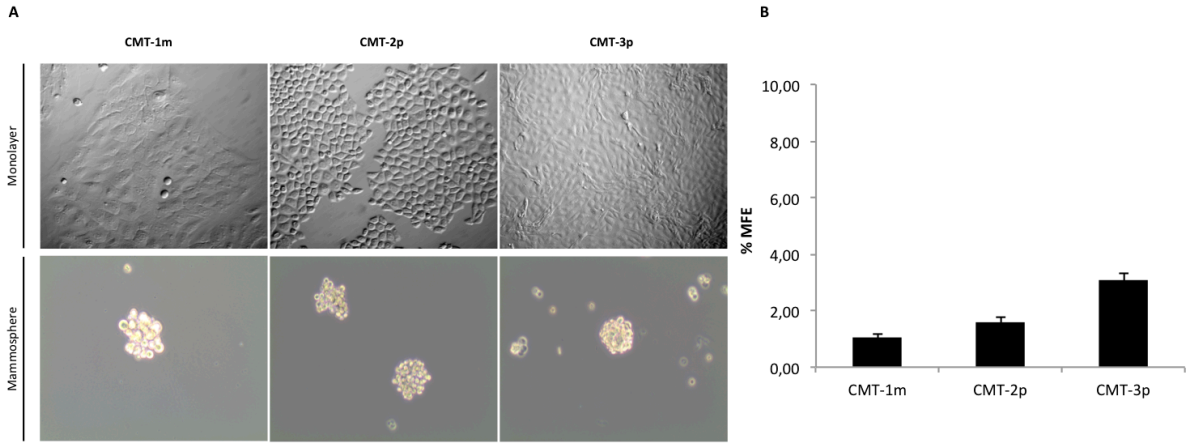


Figure 17. (A) Morphological characteristics of 2D (monolayer) and 3D mammospheres of CMT-1m, CMT-2p and CMT-3p cell lines. Monolayer magnification: 200x; Mammosphere magnification: 100x. **(B)** Percentage of mammosphere forming efficiency (MFE) of the three distinct cell lines. Each bar represents mean number ± SEM of the six independent experiments.

We also tried to characterise these mammospheres through western blot assays for some CSC markers, although without success due to low efficiency in collecting total protein lysates.

4. INVESTIGATING THE LINK BETWEEN EMT AND THE CSC PHENOTYPE IN CANINE MAMMARY TUMOUR CMT-1m, CMT-2p AND CMT-3p CELL LINES

Recent research has connected the acquisition of CSC traits with the EMT transdifferentiation program (Mani et al., 2008, Creighton et al., 2010, Hayashida et al., 2011, Li and Zhou, 2011). The EMT is a key developmental program that is often activated during cancer invasion and metastasis. In this process, cells undergo a transition form an epithelial phenotype to a more mesenchymal phenotype, acquiring abilities to invade, to resist apoptosis and to metastasize. Here, we investigated if the canine mammary tumour cell lines that present more CSC markers were the ones also expressing higher levels of EMT markers, such as fibronectin (Fib), N-Cadherin (N-Cad) and β-catenin (β-Cat).

As can be seen in Figure 18A**,** CMT-1m cell line highly express β-cat and Fib, but low levels of N-Cad; in contrast, CMT-2p cells only express β-cat, and CMT-3p highly express N-cad and β-

cat. However, in CMT-2p (Figure 18B), we observed that the localization of β-cat was not at the membrane, which can indicate that this protein is not functional in terms of adhesion.

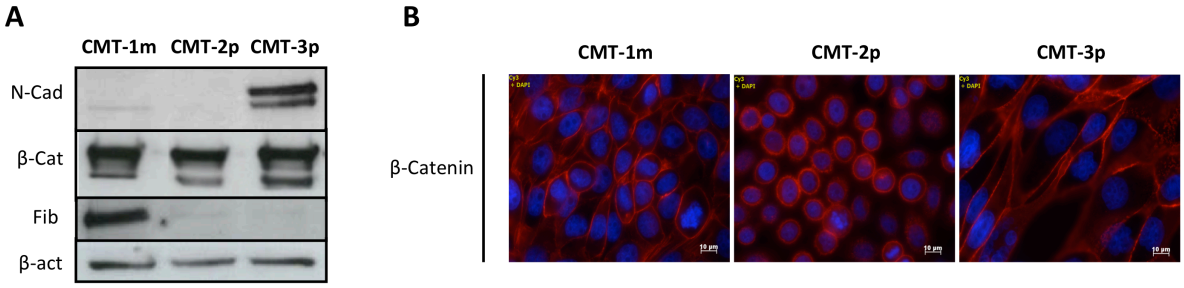


Figure 18. **(A)** Western blot showing expression of EMT markers in CMT-1m, CMT-2p and CMT-3p cell lines. CMT-1m cell line highly express β-cat and Fib, but low levels of N-Cad. On the other hand, CMT-2p only express β-cat and CMT-3p highly express N-cad and β-cat. **(B)** Immunofluorescence showed that the localization of β-cat protein is at the cellular membrane for CMT-1m and CMT-3p, but it is cytoplasmatic in CMT-2p cells. Original magnification: 630x.

5. INVESTIGATING THE EXPRESSION OF THE CANCER STEM CELL MARKERS ALDH1, CD44 AND CD24 IN A SERIES OF CANINE MAMMARY CARCINOMAS

Patients and tumour characteristics

The overall clinicopathological characteristics of our series are displayed in **Table 4.** Clinical information regarding animal age was possible in 102 cases. The mean age of dogs at the time of surgical tumour removal was 9.7 ± 2.4 years (range 4-15 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). Concerning tumour size, it was available for 98 cases. The mean maximum tumour diameter was 4.53 ± 3.99 cm, with tumours ranging from 0.5 to 20cm. As for skin ulceration, it was found in 21 (19.4%) out of the 108 cases with available information. Histologically, the 112 malignant tumour types comprised 30 tubulopapillary carcinomas (n=30; 20.7%), 30 complex carcinomas (26.8%), 25 solid carcinomas (22.3%), 14 carcinosarcomas (12.5%), 6 spindle cell carcinomas (5.4%), 4 carcinomas in benign tumour (3.6%) and 3 anaplastic carcinomas (2.7%), according to WHO criteria for canine mammary tumours (Misdorp et al., 1999). Lymphovascular invasion was present in 59 cases (52.7%). Lymph nodes were available in 53 cases with confirmed metastasis in 27 cases (50.9%). With regard to histological grade, carcinomas were classified as grade I (n=12; 10.7), grade II (n=39; 34.8) and grade III (n=61; 54.5), according to the Nottingham method.

Table 4 – Frequencies observed in the present series for clinicopathological parameters.

Clinicopathological parameters	Frequencies n(%)
Age (n=102)	
≤ 9 years old	46 (45.1)
> 9 years old	56 (54.9)
Tumour size (n=98)	
<3 cm	41 (41.8)
3-5 cm	31 (31.6)
>5 cm	26 (26.6)
Skin ulceration (n=108)	
Absent	87 (80.6)
Present	21 (19.4)
Histological type (n=112)	
Tubulopapillary carcinoma	30 (26.7)
Solid carcinoma	25 (22.3)
Carcinosarcoma	14 (12.5)
Anaplastic carcinoma	3 (2.7)
Spindle cell carcinoma	6 (5.4)
Complex carcinoma	30 (26.8)
Carcinoma in benign tumour	4 (3.6)
Lymphovascular Invasion (n=112)	
Absent	53 (47.3)
Present	59 (52.7)
Lymph node metastasis (n=53)	
Absent	26 (49.1)
Present	27 (50.9)
Histological grade (n=112)	
Grade I	12 (10.7)
Grade II	39 (34.8)
Grade III	61 (54.5)

Immunohistochemistry profiles in canine tumours

Immunohistochemical analysis was performed for CD44, CD24 and ALDH1 (CSC markers) expression in a series of canine mammary carcinomas. Evaluation of CD44 was available in 111 cases: one case was excluded due to staining absence in adjacent mammary gland (internal control), probably due to the poor preservation of the material. For CD24 and ALDH1, evaluation was performed on 102 cases, because there was no sufficient tumour material available.

In normal adjacent mammary gland, CD44 expression was found in myoepithelial cells and at the basal and some lateral surfaces of ductal and lobular epithelial cells (Figure 19A and B). Frequently, stromal tissue also showed a weak to moderate immunolabelling. CD24 expression was infrequently found in adjacent mammary gland, being characterized by luminal apical membrane and secretion staining, more pronounced in dilated ducts (Figure 20A). With regard to ALDH1, a cytoplasmic expression was focally found in ductal luminal epithelial cells of adjacent mammary gland, occasionally associated with nuclear expression (Figure 21A).

The results of the immunohistochemical analysis for CD44 and CD24 are shown in Table 5 and 6**,** when considering four or two categories, respectively, according with the evaluation criteria previously described. Concerning CD44 membrane staining, 81.1% (90/111) of the cases were considered positive, with 56 cases (50.5%) showing more than 50% of positive neoplastic cells (Figure 19C and D). In contrast, for membrane CD24, a minority of cases (4.9%, 5/102) were classified as positive, being characterized by a clear membrane expression in more than 25% of neoplastic cells (Figure 20B, C and D). Cytoplasmic staining was also considered for CD24 marker, with 9.8% (10/102) of positive carcinoma cases.

Table 5 – Immunoexpression observed for CD44 and CD24.

	<10%	**10-25%**	**26-50%**	**>50%**
CD44 (n=111)	21 (18.9)	13 (11.7)	21 (18.9)	56 (50.5)
CD24 (n=102)	79 (77.5)	18 (17.6)	5 (4.9)	0 (0.0)

Table 6 – Immunoexpression divided in two major categories for CD44 and CD24.

	Negative	**Positive**
CD44 (n=111)	21 (18.9)	90 (81.1)
CD24 (n=102)		
Membrane	97 (95.1)	5 (4.9)
Cytoplasm	92 (90.2)	10 (9.8)

Immunohistochemical staining for ALDH1 was classified in two categories both at epithelial tumour cells and stromal cells (Table 7). A clear ALDH1 cytoplasmic expression was observed in more than 1% of tumour cells in 36 (35.3%) carcinoma cases (Figure 21B and C). Nine of these positive cases (9/36; 25%) also showed nuclear positivity. In addition, moderate/strong stromal staining for ALDH1 was observed in 22.5% (23/102) cases (Figure 21D).

Table 7 – Immunoexpression observed for ALDH1.

	Epithelial		**Stromal**	
	Negative	Positive	None/weak	Moderate/strong
ALDH1 (n=102)	66 (64.7%)	36 (35.3%)	79 (77.5%)	23 (22.5%)

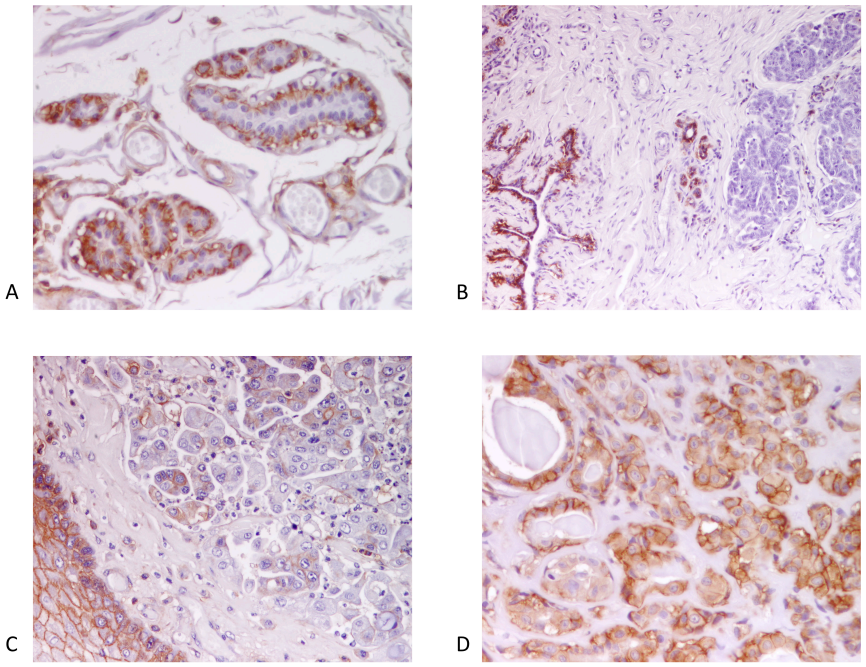


Figure 19. Immunohistochemical expression of CD44 in canine mammary tissues. **(A)** Adjacent mammary gland showing CD44 immunoexpression in the myoepithelial cell layer; **(B)** Negative immunoexpression in tumour epithelial cells (on the right); **(C)** Tubulopapillary carcinoma with neoplastic epithelial cells weakly positive for CD44**; (D)** Tubulopapillary carcinoma with neoplastic epithelial cells strongly positive for CD44. Original magnification: 400x (A, C and D) and 200x (B).

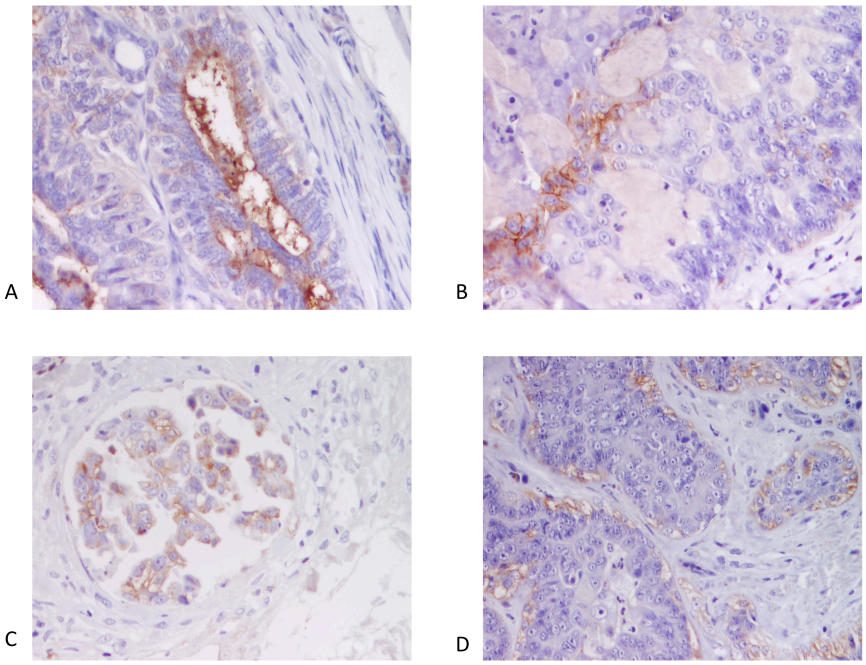


Figure 20. Immunohistochemical expression of CD24 in canine mammary tissues. **(A)** An apically accentuated membranous staining of luminal ductal epithelial cells in adjacent mammary gland; **(B)** Tubulopapillary carcinoma with neoplastic epithelial cells showing CD24 expression; **(C)** Neoplastic embolus weakly positive for CD24; **(D)** Carcinoma with basally located neoplastic cells positive for CD24, which are in direct contact with the stroma. Original magnification: 400x.

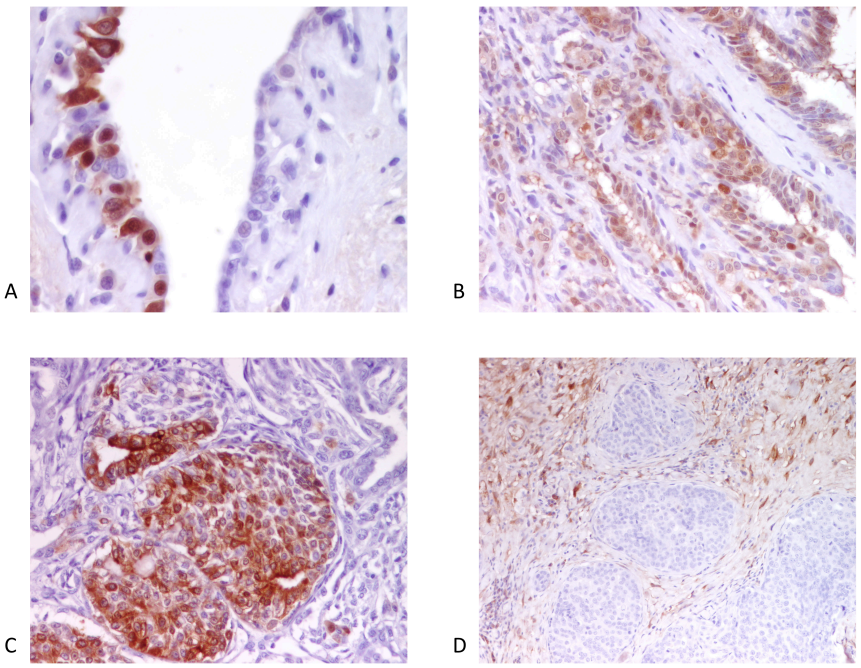


Figure 21. Immunohistochemical expression of ALDH1 in canine mammary tissues. **(A)** Cytoplasmic and nuclear expression of ALDH1 in luminal ductal epithelial cells of adjacent mammary gland; **(B)** Complex carcinoma with neoplastic epithelial cells showing ALDH1 cytoplasmic and nuclear expression; **(C)** Complex carcinoma showing cytoplasmic expression by neoplastic epithelial cells; **(D)** Solid carcinoma, characterized by moderate stromal expression of ALDH1. Original magnification: 600x (A); 400x (B and C) and 200x (D).

Association of CSC markers with clinicopathological parameters

In the present study, CSC marker expression was associated with several classical clinicopathological parameters, as well as with Ki-67 index (Table 8). CD44 expression was significantly associated with the histological type ($p<0.001$), with all tumour types evaluated exhibiting more than 65% of positive cases, with the exception of solid carcinoma type. This tumour type was negative in more than half cases (n=13; 54.2%). No significant associations were found for other parameters, although there is a tendency towards significance with regard to histological grade, with all grade I carcinomas positive for CD44 compared to grade III, with 75% positive cases.

No significant associations were found between CD24 expression and clinicopathological parameters evaluated, possibly due to the small number of positive cases. However, positive CD24 cases consisted in the following histological types: two tubulopapillary carcinomas, one solid carcinoma, one carcinosarcoma and one complex carcinoma. These tumours were classified as three grade II and two grade III carcinomas.

Concerning ALDH1 cytoplasmic expression, it was significantly associated (p=0.001) with the absence of lymphovascular invasion. Only eleven out of 54 cases (20.4%) with vascular invasion showed ALDH1 expression, whereas 25/48 carcinomas (52.1%) without vascular invasion were immunopositive for this molecule. ALDH1 was also associated with histological type (p=0.045), being frequently positive in tubulopapillary and complex carcinoma.

With regard to stromal ALDH1 expression, no significant associations were found between its expression and clinicopathological variables (Table 9).

Table 8 – Association between CSC markers and clinicopathological parameters and biological biomarker.

Clinicopathological parameters	CD44 (n (%))			CD24 (n (%))			ALDH1 (n (%))		
	n	Negative	Positive	n	Negative	Positive	n	Negative	Positive
Age	102	19	83	94	89	5	94	62	32
≤ 9 years old	50	8 (16.0)	42 (84.0)	47	45 (95.7)	2 (2.1)	62	29 (61.7)	18 (38.3)
> 9 years old	52	11 (21.2)	41 (78.8)	47	44 (93.6)	3 (3.2)	32	33 (70.2)	14 (29.8)
p Value		0.613			1.000			0.514	
Tumour size	98	21	77	92	87	5	92	59	33
<3 cm	39	5 (12.8)	34 (87.2)	37	34 (91.9)	3 (8.1)	37	26 (70.3)	11 (29.7)
3-5 cm	27	7 (25.9)	20 (74.1)	25	24 (96.0)	1 (4.0)	25	13 (52.0)	12 (48.0)
>5 cm	32	9 (28.1)	23 (71.9)	30	29 (96.7)	1 (3.3)	30	20 (66.7)	10 (33.3)
p Value		0.235			0.731			0.337	
Skin ulceration	107	21	86	100	95	5	100	65	35
Absent	87	18 (20.7)	69 (79.3)	80	77 (96.3)	3 (3.8)	80	49 (61.2)	31 (38.8)
Present	20	3 (15.0)	17 (85.0)	20	18 (90)	2 (10)	20	16 (80.0)	4 (20.0)
p Value		0.758			0.261			0.189	
Histological type	111	21	90	102	97	5	102	66	36
Tubulopapillary carcinoma	30	4 (13.3)	26 (86.7)	29	27 (93.1)	2 (6.9)	29	15 (51.7)	14 (48.3)
Solid carcinoma	24	13 (54.2)	11 (45.8)	22	21 (95.5)	1 (4.5)	23	19(82.6)	4 (17.4)
Carcinosarcoma	14	2 (14.3)	12 (95.7)	12	11 (91.7)	1 (8.3)	12	10 (83.3)	2 (16.7)
Anaplastic carcinoma	3	1 (33.3)	2 (66.7)	3	3 (100)	0 (0.0)	3	3 (100)	0 (0.0)
Spindle cell carcinoma	6	0 (0.0)	6 (100)	5	5 (100)	0 (0.0)	4	3 (75.0)	1 (25.0)
Complex carcinoma	30	1 (3.3)	29 (96.7)	27	26 (96.3)	1 (3.7)	27	13 (48.1)	14 (51.9)
Carcinoma in benign tumour	4	0 (0.0)	4 (100)	4	4 (100)	0 (0.0)	4	3 (75.0)	1 (25.0)
p Value		**<0.001**			1.000			**0.045**	
Lymphovascular Invasion	111	21	90	102	97	5	92	66	36
Absent	53	9 (17.0)	44 (83.0)	49	46 (93.9)	3 (6.1)	48	23 (47.9)	25 (52.1)
Present	58	12 (20.7)	46 (79.3)	53	51 (96.2)	2 (3.8)	54	43 (79.6)	11 (20.4)
p Value		0.638			0.669			**0.001**	
Lymph node metastasis	52	8	44	48	43	5	49	32	17
Absent	25	3 (12.0)	22 (88.0)	25	22 (88.0)	3 (12.0)	25	15 (60.0)	10 (40.0)
Present	27	5 (18.5)	22 (81.5)	23	21 (91.3)	2 (8.7)	24	17 (70.8)	7 (29.2)
p Value		0.705			1.000			0.310	
Histological grade	111	21	90	102	97	5	102	66	36
Grade I	12	0 (0)	12 (100)	11	11 (100)	0 (0.0)	10	6 (60.0)	4 (40.0)
Grade II	39	6 (15.4)	33 (84.6)	36	33 (91.7)	3 (8.3)	36	19 (52.8)	17 (47.2)
Grade III	59	15 (25.0)	45 (75.0)	55	53 (96.4)	2 (3.6)	56	41 (73.2)	15 (26.8)
p Value		0.117			0.406			0.126	
Median Ki-67	72	26.89	21.93	65	21.35	32.50	65	23.93	20.53
(Min-Max)		12.10-56.36	5.39-49.20		5.39-56.36	24.42-40.58		7.77-56.36	5.39-36.85
p Value		0.261			0.239			0.170	

Table 9 – Association of stromal ALDH1 markers with clinicopathological parameters.

Clinicopathological parameters	Stromal ALDH1 (n (%))		
	N	Negative	Positive
Age			
≤ 9 years old	47	36 (76.6)	11 (23.4)
> 9 years old	47	38 (80.9)	9 (19.1)
p Value		0.802	
Tumour size			
<3 cm	37	32 (86.5)	5 (13.5)
3-5 cm	25	17 (68.0)	8 (32.0)
>5 cm	30	20 (66.7)	10 (33.3)
p Value		0.112	
Skin ulceration			
Absent	80	61 (76.3)	19 (23.8)
Present	20	16 (80.0)	4 (20.0)
p Value		1.000	
Histological type			
Tubulopapillary carcinoma	27	19 (70.4)	8 (29.6)
Solid carcinoma	22	16 (72.7)	6 (27.3)
Carcinosarcoma	12	10 (83.3)	2 (16.7)
Anaplastic carcinoma	3	2 (66.7)	1 (33.3)
Spindle cell carcinoma	4	4 (100)	0 (0.0)
Complex carcinoma	27	21 (77.8)	6 (22.2)
Carcinoma in benign tumour	4	4 (100)	0 (0.0)
p Value		0.748	
Lymphovascular Invasion			
Absent	48	39 (81.2)	9 (18.8)
Present	52	38 (73.1)	14 (26.9)
p Value		0.353	
Lymph node metastasis			
Absent	24	21 (87.5)	3 (12.5)
Present	23	16 (69.6)	7 (30.4)
p Value		0.168	
Histological grade			
Grade I	10	8 (80.0)	2 (20.0)
Grade II	36	25 (69.4)	11 (30.6)
Grade III	53	43 (81.1)	10 (18.9)
p Value		0.445	
Median Ki67 index		23.4	22.25
(Min-Max)		(5.39-56.36)	(16.76-41.53)
p Value		0.78	

When the association between the combined expression of $CD44^{+}/CD24^{-}/ALDH1^{+}$ and clinicopathological data was addressed (Table 10), we observed that the presence of the CSC phenotype was significantly associated with histological type, namely tubulopapillary and complex carcinomas.

Table 10 – Association between the combined expression of $CD44^+/CD24^-/ALDH1^+$ with clinicopathological parameters.

Clinicopathological parameters	N	CSC phenotype ($CD44^+/CD24^-/ALDH1^+$)	
		Negative	Positive
Age			
≤ 9 years old	47	32 (68.1)	15 (31.9)
> 9 years old	46	36 (78.3)	10 (21.7)
p Value		0.351	
Tumour size			
<3 cm	36	29 (80.6)	7 (19.4)
3-5 cm	25	17 (68.0)	8 (32.0)
>5 cm	29	20 (69.0)	9 (31.0)
p Value		0.452	
Skin ulceration			
Absent	79	57 (72.2)	22 (27.8)
Present	19	15 (78.9)	4 (21.1)
p Value		0.773	
Histological type			
Tubulopapillary carcinoma	27	18 (66.7)	9 (33.3)
Solid carcinoma	20	19 (95.0)	1 (5.0)
Carcinosarcoma	12	11 (91.7)	1 (8.3)
Anaplastic carcinoma	3	3 (100)	0 (0.0)
Spindle cell carcinoma	4	3 (75.0)	1 (25.0)
Complex carcinoma	27	14 (51.9)	13 (48.1)
Carcinoma in benign tumour	4	3 (75.0)	1 (25.0)
p Value		**0.019**	
Lymphovascular Invasion			
Absent	48	31 (64.6)	17 (35.4)
Present	50	41 (82.0)	9 (18.0)
p Value		0.068	
Lymph node metastasis			
Absent	23	16 (69.6)	7 (30.4)
Present	22	16 (72.7)	6 (27.3)
p Value		1.000	
Histological grade			
Grade I	10	6 (60.0)	4 (40.0)
Grade II	36	24 (66.7)	12 (33.3)
Grade III	51	41 (80.4)	10 (19.6)
p Value		0.247	
Median Ki-67	67	23.11	20.96
(Min-Max)		(7.7-56.36)	(5.39-36.85)
p Value		0.255	

Kaplan-Meier survival curves were generated in order to assess the potential impact of the three CSC markers (CD44, CD24, ALDH1) in disease-free and overall survival (Figure 22 and 23).

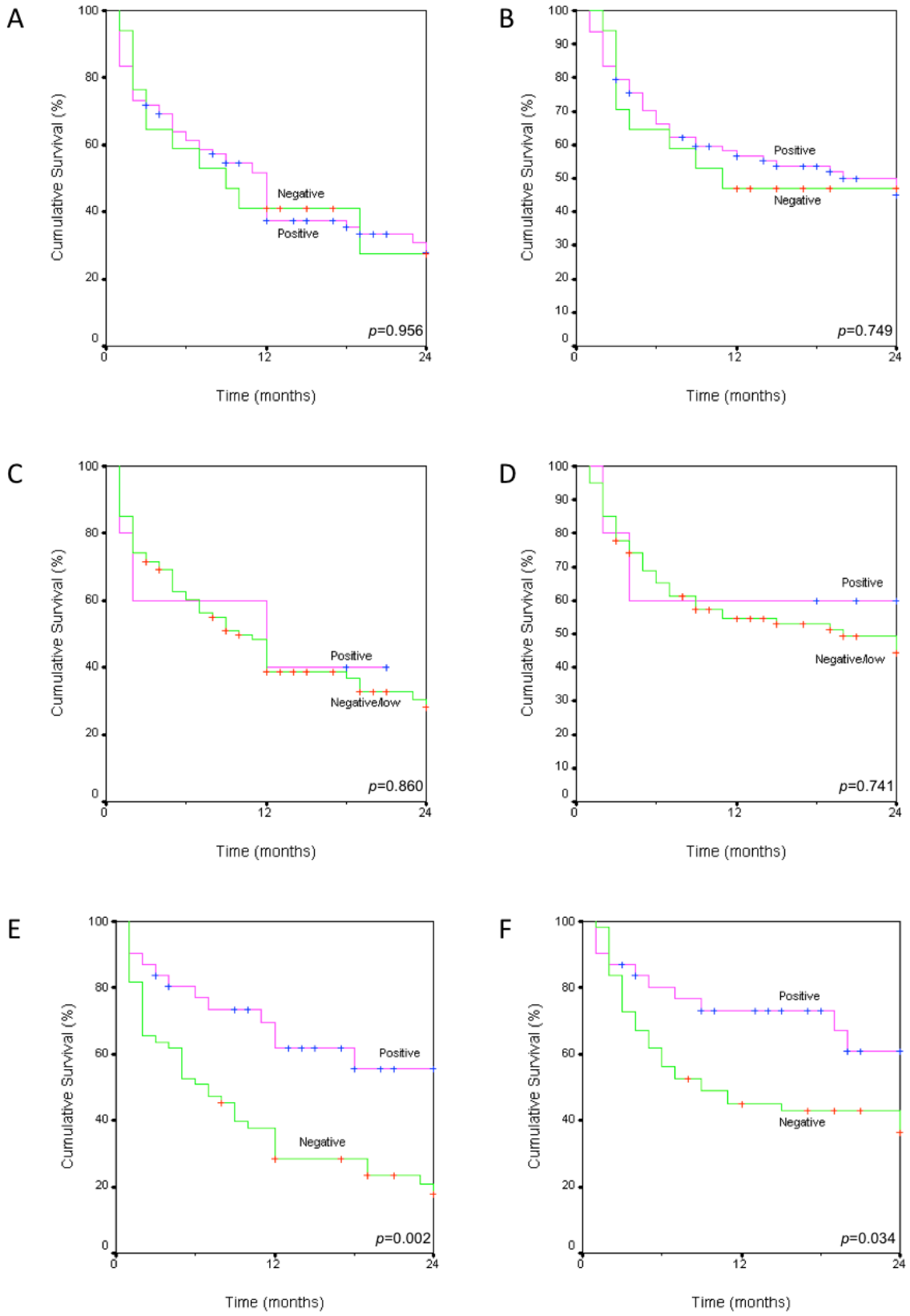


Figure 22. Kaplan-Meier disease-free survival **(A) (C) (E)** and overall survival **(B) (D) (F)** for the expression of CD44, CD24 and ALDH1, respectively.

With the exception of epithelial ALDH1 expression, no significant differences were observed. ALDH1 reach statistically significant levels, meaning that the absence of this protein was a significant predictor of DFS (p=0.002) and OS (p=0.034) in dogs bearing mammary carcinomas (Figure 22E and F). On the other hand, stromal positivity was not associated with survival (Figure 23).

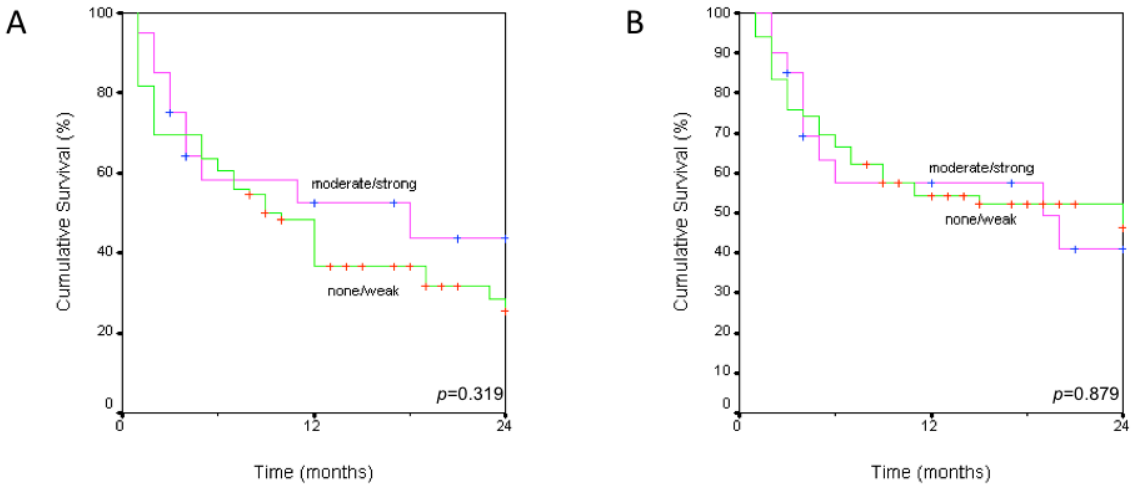


Figure 23. Kaplan-Meier disease-free survival **(A)** and overall survival **(B)** for the expression of stromal ALDH1.

Similarly, when considering the influence on survival of the presence of the CSC phenotype ($CD44^{+}CD24^{-}ALDH1^{+}$), no statistically significant differences were found (Figure 24).

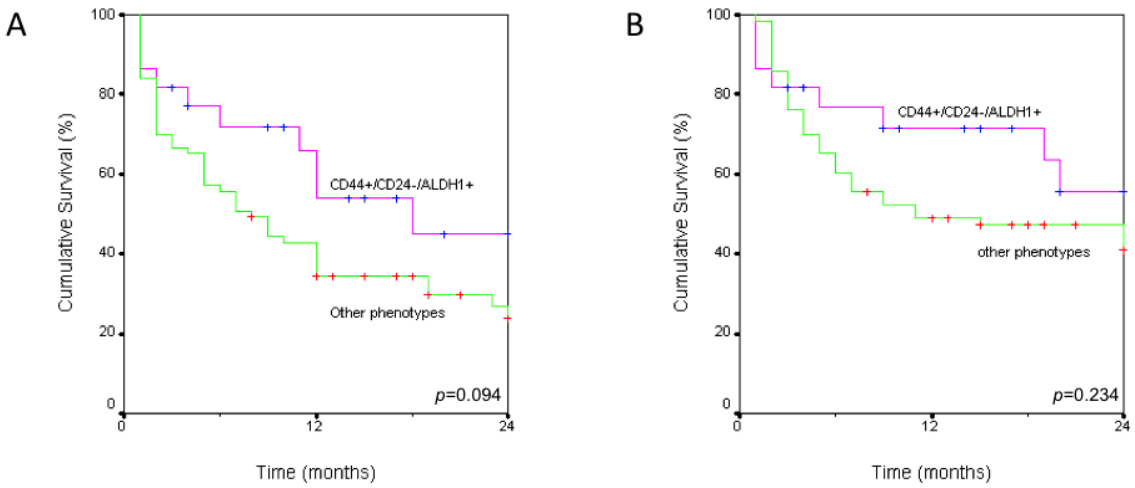


Figure 24. Kaplan-Meier disease-free survival **(A)** and overall survival **(B)** for the combination of $CD44^{+}CD24^{-}ALDH1^{+}$ and other phenotypes.

DISCUSSION

1. CHARACTERISATION OF THE PHENOTYPE AND FUNCTION OF THE CANINE MAMMARY TUMOUR CELL LINES CMT-1m, CMT-2p AND CMT-3p.

Cell lines have been used as *in vitro* models for several years in cancer research to study the biology of human neoplastic cells. These models allow us to work in highly controlled conditions, being unconstrained by the practical and ethical considerations necessary with *in vivo* models (Pinho et al., 2012). Despite cell lines are not exactly representative of the *in vivo* cancer cell population, these might be the closest equivalent to the tumour initiating cells (or the origin of cancers), or even to the CSCs, offering simplified systems for drug testing, as well as models for the study of phenotype and genotype evolution under selective pressure (van Staveren et al., 2009).

However, there are still few available cell cultures to use in veterinary sciences, despite the considerable growth of interest on this methodology in the last years. Based on this fact, and in order to contribute with important and additional tools for canine mammary biopathological research, we decided to establish three spontaneous canine mammary tumour cell lines to use in the present work: CMT-1m, established from a pulmonary metastasis from a canine mammary tumour, and CMT-2p and CMT-3p, established from primary canine mammary carcinomas.

Firstly, a characterisation of these canine mammary tumour cell lines through immunofluorescence and western blot analysis was performed, aiming to investigate the expression of some molecular markers and functional activities.

With regard to hormonal receptors (ER and PgR), all cell lines were positive for ER, although CMT-3p showed lower levels, whereas no cell line revealed PgR positivity. It is well known that estrogen and ER play essential roles in both normal breast development and breast cancer progression (Pearce and Jordan, 2004). Estrogen exerts its biological effects usually by binding to ER, which mainly exists in the nucleus as a member of the nuclear receptor superfamily of transcription factors. The estrogen–ER complex, through genomic and nongenomic pathways, leads to cellular proliferation and differentiation (Murphy and Watson, 2002, Yamaguchi, 2007). Additionally, PgR is an estrogen-regulated gene and its expression is therefore thought to indicate a functioning ER pathway (Gama et al., 2008a); thus, its absence of expression in these three cell lines might indicate a non-functional ER signalling.

Although in human breast cancer, hormone receptor status is currently evaluated to estimate prognosis and in predicting responsiveness to endocrine treatment, in canine cancer there is still insufficient data in the literature regarding its prognostic significance. Absence of hormonal receptors (ER and PgR) is usually associated with poor survival in canine victims of

mammary cancer (Rutteman et al., 1988, Gama et al., 2008b), being frequently coupled with the expression of basal/myoepithelial cell markers, such as p63 or P-cadherin (Gama et al., 2008b). However, these cell lines were negative for these basal markers, as well as for HER2, a receptor tyrosine kinase that is overexpressed in about 15–30% of human breast carcinomas and highly correlated with aggressive clinicopathological features (Burstein, 2005).

As human breast cancer, canine mammary cancer is a molecularly heterogeneous disease, being important to have available specific cancer cell lines that can reflect that molecular diversity. Microarray based studies have identified four distinct molecular subtypes among human breast carcinomas: luminal A, luminal B, HER2-overexpressing and basal-like, showing characteristic gene-expression patterns and diverse clinical outcomes. Based on our findings, these canine mammary tumour cell lines, particularly CMT-1m and CMT-2p, might be classified as luminal cell lines, given their strong positivity for ER, which can be putatively used in future studies, namely in the search of endocrine therapies.

Several other molecules might be considered relevant markers to evaluate the molecular profile and aggressive behaviour of human and canine malignancies, like adhesion receptors, such as E-cadherin or P-cadherin deregulation. Loss or down-regulation of E-cadherin/β-catenin complexes has been associated with tumour progression in human (Gamallo et al., 1993, Yoshida et al., 2001) and canine mammary cancer (Gama et al., 2008b), as well as anomalous epithelial overexpression of P-cadherin in human breast cancer was associated with an aggressive biological behaviour and poor patient outcome (Paredes et al., 2002, Paredes et al., 2005), which has not been found in the canine model, although its frequent overexpression in invasive canine mammary carcinomas (Gama et al., 2004).

Interestingly, although all cell lines showed a positive expression for E-cadherin, only CMT-1m and CMT-3p showed membrane localization, while CMT-2p showed a cytoplasmic expression pattern. This observation led us to consider that CMT-2p E-cadherin was probably not functional, which was later on confirmed by the aggregation assay. In fact, in the context of cancer, E-cadherin has traditionally been categorized as a tumour suppressor, given its essential role in the formation of proper intercellular junctions, and its downregulation in the process of epithelial-mesenchymal transition (EMT) in epithelial tumour progression. Germline or somatic mutations in the E-cadherin gene (CDH1) or downregulation by epigenetic mechanisms have been described in a small subset of epithelial cancers. However, recent evidence also points towards a promoting role of E-cadherin in several aspects of tumour progression (Paredes et al., 2012). This includes preserved (or increased) E-cadherin expression in microemboli of inflammatory breast carcinoma, a possible "mesenchymal to epithelial transition" (MET) in ovarian carcinoma,

collective cell invasion in some epithelial cancers, a recent association of E-cadherin expression with a more aggressive brain tumour subset, as well as the intriguing possibility of E-cadherin involvement in specific signalling networks in the cytoplasm and/or nucleus (Rodriguez et al., 2012). Uyama et al. (2006), for example, evaluated E-cadherin expression in canine mammary cell lines derived from primary and metastatic tissues, demonstrating that metastatic derived cell lines exhibited higher aggregation indices, when compared to primary derived cell lines, similarly to our findings. In fact, CMT-1m showed more compact aggregates, which suggests that metastatic cells might recover E-cadherin function once they reach the metastatic niche (Uyama et al., 2006).

Moreover, it was interesting to see that, functionally, CMT-3p cell line, which was membrane E-cadherin positive, was the one with higher rates of proliferation, cell death and invasive capacity, whereas CMT-2p, which showed non-functional E-cadherin, showed the lower levels of any of these evaluated properties. Additionally, by β-casein and gelatin zymography, we found that MMP-7 and MMP-9 activity was more evident in CMT-3p cell line, whereas CMT-1m and CMT-2p showed higher levels of MMP-1 and MMP-2. Indeed, it has been shown that MMP-7 activity, a protease implicated in mammary tumour growth, is responsible for the proliferative effects in Madin-Darby canine kidney (MDCK) cells (Harrell et al., 2004), as well as studies with MMP-7 null mice provided support for its role in the potentiation of epithelial cell apoptosis (Powell et al., 1999). Additionally, it has been demonstrated that MMP-7 can cleave the extracellular portion of E-cadherin, demonstrating the mechanism by which it can directly impact loose and irregular cell-cell contacts, increased cell migration, loss of epithelial cell polarization and increased cell proliferation via RhoA activation (Lynch et al., 2010). Based on this data concerning MMP-7, we can in part explain why CMT-3p cells, despite being E-cadherin positive, show high levels of proliferation, apoptosis and invasion. Regarding MMP-9, also mainly found in CMT-3p cells, it has been described that it can play an important role in malignant canine mammary tumours, being their levels determination suggested as a feasible method for detecting neoplastic growth in dogs (Aresu et al., 2011).

Concerning MMP-2 and MMP-1, it has been shown that, when these two proteases are co-expressed in human breast cancer cells, these facilitate the assembly of new tumor blood vessels, the release of tumor cells into the circulation, and the breaching of lung capillaries by circulating tumor cells to seed pulmonary metastasis (Gupta et al., 2007). Indeed, metastasis capacity entails numerous biological functions that collectively enable cancerous cells from a primary site to disseminate and overtake distant organs. This can mean that, although CMT-1m

and CMT-2p cell lines are less-invasive in Matrigel *in vitro* assays, they can have high metastatic potential, which needs to be further evaluated *in vivo*.

Thus, based on the results obtained, and taking the literature into account, CMT-3p seems to be the cell line with the most aggressive phenotype and *in vitro* functional behaviour, showing characteristics highly related with mesenchymal-like cells, despite the membrane E-cadherin expression. In contrast, CMT-1m and CMT-2p, although not invasive *in vitro*, showed high levels of MMP1 and MMP2, which can indicate their high capacity to metastasize *in vivo* to distant sites, which needs to be further clarified in the future.

2. INVESTIGATING THE CANCER STEM CELL PHENOTYPE OF THE CANINE MAMMARY TUMOUR CELL LINES CMT-1m, CMT-2p AND CMT-3p

Cancer cells and stem cells share many phenotypic and functional similarities, such as the prolonged self-renewal or immortality, high proliferative capacity, and the propensity for tissue migration. These similarities have led to the hypothesis that tumours were probably organized in a hierarchy of heterogeneous cell populations with different biological properties, as stem or progenitor cells in normal tissues, and that the capability to sustain tumour formation and growth was residing only in a small proportion of tumour cells. Such cells are called CSCs and are considered to be critically important for tumour proliferation, survival, invasion and metastasis (Charafe-Jauffret et al., 2008, Ablett et al., 2012).

Indeed, this small population of tumour cells has been identified in several cancers, including human breast cancer, and several markers have been defined to select cells with stem-cell properties, as CD44, CD24, ALDH1, α6 and β4 integrins (Al-Hajj et al., 2003, Ponti et al., 2005, Stingl et al., 2006, Ginestier et al., 2007, Pontier and Muller, 2009, Vieira et al., 2012). Here, in this work, we could observe that CMT1-m and CMT-2p cell lines were the ones showing an increased expression of human mammary stem-cell related markers, using western blot and immunofluorescence. Indeed, these cell lines showed positivity for both integrins, to ALDH1 and to CD44v6. In contrast, CMT-3p cells were completely negative or showed low levels of all these markers. However, concerning CD44s, only CMT-1m and CMT3-p showed positive expression, while CMT-2p presented low levels, only detected by immunofluorescence.

Taking this distribution into account, and on the fact that CMT-2p was the cell line presenting low invasive ability, it seems that our results are in accordance with the recent study of Brown et al. (2011) that demonstrated, using *in vivo* and *in vitro* models, that there is a shift in CD44v expression to the standard isoform (CD44s) during the acquisition of the mesenchymal

phenotype and, consequently, during the EMT process that facilitates the invasion capacity in the Matrigel extracellular matrix. These authors also refer the upregulated expression of CD44s in high-grade human breast tumours (Brown et al., 2011). On the other hand, there are studies showing the association between CD44s expression and cell proliferation in canine mammary tumour cell lines (Blacking et al., 2011), which also is very well correlated with the proliferation results obtained with our canine mammary cancer cell lines. Interestingly, an immunohistochemical study showed that CD44s expression was more upregulated in benign canine mammary tumours than in malignant ones (Paltian et al., 2009).

In addition, using flow cytometry, we investigated the presence of the human CSC phenotype in our CMT cell lines, using the combined expression of the cell surface markers CD44 and CD24 and the ALDEFLUOR assay. Interestingly, we could observe that CMT-1m was enriched in the $CD44^{+}/CD24^{+}$ cell population (>90%), being CMT-2p and CMT-3p highly enriched for the human $CD44^{+}/CD24^{low}$ breast CSC phenotype (near 50%). Although ALDH1 expression was only detected in CMT-2p cells by western blot, similar levels of ALDH1 activity were found for the three cell lines using the ALDEFLUOR assay (between 4-7%); this result can be explained, since the ALDEFLUOR assay detects the activity of several isoforms of ALDH1, while western blot only allows the detection of the ALDH1A1 expression. For the future, it will be interesting to develop similar ALDEFLUOR assays using different but more specific substrates that will allow the detection of the exact ALDH isoenzymes expressed in any given cell type (Moreb et al., 2012).

Based on these expression results, we can confirm that CMT-3p cells are indeed the ones harbouring a more aggressive *in vitro* phenotype, despite the negative expression for the stem-cell related markers. These cells show a more mesenchymal phenotype and harbour high levels of cells with the CSC phenotype $CD44^{+}CD24^{-/low}$ and ALDH1 activity. All these results can explain the high proliferation and invasion capacity of this specific cell line.

Concerning CMT-2p, due to their strong expression of stem-cell related markers, could mean that this particular tumour was originated from putative normal mammary stem cells, but did not acquire enough mutations to permit the acquisition of a high invasive capacity in *in vitro* assays. Additionally, their phenotype can be more related with an efficient metastatic cell line, due to the possible cross-talk with the tumour microenvironment, than with a high invasive phenotype. CMT-1m seems to harbour a mixed phenotype between CMT-2p and CMT-3p.

Very recently, there is a study, in canine mammary tumours, that showed the importance of ALDH1 activity in sphere-forming assays (that measure self-renewal capacity) since, in its absence, the cell lines were not able to form spheres. In the same study, the authors also showed that the population of cells with ALDH1 activity were enriched in a $CD44^{+}/CD24^{-}$ population and

only $1x10^4$ cells with ALDH1 activity were sufficient for tumour formation in all injected mice (Michishita et al., 2012). Although in our study we were not able to perform the gold standard *in vivo* tumorigenic assays in nude mice, inoculating different cell dilutions of the different cancer cell populations sorted by CD44/CD24 or ALDH1 activity, we were able to evaluate the efficiency of the different cell lines in forming mammospheres.

3. STUDYING THE ABILITY OF THE CMT-1m, CMT-2p AND CMT-3p CANINE MAMMARY TUMOUR CELL LINES TO GROW IN ANCHORAGE INDEPENDENT CONDITIONS AS SPHEROIDS

Dontu et al. (2003) have developed an *in vitro* cultivation system that allows the propagation of human mammary epithelial cells in undifferentiated state, based on the ability of cells to proliferate in suspension, as mammospheres. These authors also demonstrated that spheres were enriched in early progenitor/stem cells (Dontu et al., 2003). Because of that, in the field of breast cancer research, this *in vitro* cultivation system represents the most suitable method for growing and enriching cultures for candidate tumour initiating stem cells, which normally harbour stem cell-like properties such as $CD44^+CD24^{-/low}$ (Ponti et al., 2005) and $ALDH^+$ (Ginestier et al., 2007, Charafe-Jauffret et al., 2009b) phenotypes.

This methodology has already been used in canine mammary tumour cell lines and was showed that cells obtained from spheres that display self-renewing properties, have multi-lineage differentiation potential, could generate complex branched tubular structures *in vitro* (Cocola et al., 2009) and demonstrate high capacity to form tumours *in vivo* (Cocola et al., 2009, Michishita et al., 2010).

Based on the phenotype evaluated for the three cell lines that we were working with, it would be expectable that CMT-2p and CMT-3p would be the ones with more ability to grow in the referred conditions. In fact, we found that all the cell lines showed capacity to grow as spheroids, although CMT-3p was the one presenting the higher MFE. These results in canine mammary tumour cell lines, make us believe that there is no relation between the expression of human stem cell-related markers, such as α6- and β4-integrins, and the capacity of these cell lines to grow as mammospheres. However, it seems to occur an association between the ALDH1 activity and $CD44^+/CD24^{low}$ phenotype and the capacity to generate mammospheres. Indeed, some preliminary results showed that CMT-1m cells were not capable to form new spheres in a second generation. This fact is in accordance with the study that referred the importance of the CSC phenotype $CD44^+/CD24^{low}$ to generate spheres in anchorage independent conditions (Ponti et al., 2005).

4. INVESTIGATING THE LINK BETWEEN EMT AND THE CSC PHENOTYPE IN CANINE MAMMARY TUMOUR CMT-1m, CMT-2p AND CMT-3p CELL LINES

Recent observations imply that there is a crosstalk between EMT and CSC properties (Mani et al., 2008, Creighton et al., 2010, Hayashida et al., 2011, Li and Zhou, 2011). EMT is a multistep process in which cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility (Li and Zhou, 2011). This process is ultimately thought to promote cancer cell progression and invasion (Shipitsin et al., 2007, Creighton et al., 2010, Hayashida et al., 2011).

A hallmark of EMT is the loss of E-cad expression that has been associated with undifferentiated breast carcinomas and tumour progression both in human and canine mammary cancer (Gamallo et al., 1993, Yoshida et al., 2001, Knudsen and Wheelock, 2005, Gama et al., 2008b). Recently, it was also shown that activation of the β-catenin pathway is required for the maintenance of EMT-associated stem cell-like properties (Li and Zhou, 2011). Additionally, it was shown that the most highly invasive carcinoma cell lines, that lose E-cadherin, upregulate N-cadherin (Hazan et al., 1997). Indeed, forced expression of N-cadherin in E-cadherin positive breast cancer cell lines induced an invasive phenotype without supressing E-cadherin expression (Nieman et al., 1999, Hazan et al., 2000). More recently, in a study that demonstrated that CD44s expression was upregulated in high-grade human breast tumours, a correlation with the level of the mesenchymal marker N-cadherin was also established (Brown et al., 2011).

Here, in our work, in order to confirm our idea that CMT-3p was the cell line harbouring a strong EMT phenotype, we decided to study N-cadherin and β-catenin expression. We confirmed that CMT-3p is positive for the expression of N-cadherin and β-catenin. Coincidently or not, this cell line was also the one that showed high levels of CD44s expression, MFE, proliferation and invasive ability. Regarding CMT-1m, we observed the expression of CD44s, β-catenin, fibronectin, and N-cadherin in very low levels. Concerning CMT-2p, the cell line that present almost no cells with invasive capacity, there was no expression of mesenchymal markers, which seem to be relevant for the invasion process.

Although these results need to be further explored, it seems that EMT, which has been highly implicated as the critical event initiating cancer invasion and metastasis, is not so associated with stem-cell traits that some cancer cell populations maintain in high levels, as occur with CMT-2p cells. Similar to actors changing costumes during a performance, it is clear that cancer cells undergo many rapid changes during the process of tumour progression, including EMT, acquisition of CSC properties, and MET. Such changes allow the tumour to compete with the

normal microenvironment to overcome anti-tumorigenic pressures. Then, once tissue homeostasis is lost, the altered microenvironment can itself become a potent tumour promoter. Thus, it is needed to study the changes that cancer cells undergo in converting from EMT to CSCs in a tumour microenvironment, to understand the mechanisms behind invasion and metastasis and provide insights into prevention of metastasis.

5. INVESTIGATING THE EXPRESSION OF THE CANCER STEM CELL MARKERS ALDH1, CD44 AND CD24 IN A SERIES OF CANINE MAMMARY CARCINOMAS

Although cell lines constitute a valid model in cancer research, *in vitro* cell growth might result in a selection of clones with a given set of gene expressions, phenotypical characteristics, and functions adapted to *in vitro* conditions. Furthermore, cell lines miss the important role that the tumour microenvironment has in cancer biology and progression (Pinho et al., 2012). Keeping this in mind, and based on our findings in canine cancer cell lines, we thought to investigate the relevance of the CSC markers (ALDH1, CD44 and CD24) expression in a series of primary tumours of the canine mammary gland. To the best of our knowledge, there are no studies addressing the expression of these markers in canine mammary tissues, with the exception of CD44. Even in human breast cancer, a small number of studies identified the $CD44^{+}CD24^{-/low}ALDH^{+}$ CSC phenotype in primary tumours, with investigations mainly performed in cell cultures or murine models (Al-Hajj et al., 2003, Ponti et al., 2005).

As shown in the results section, CD44 was commonly expressed among primary carcinomas (81.1% of positive cases), whereas expression of CD24 and ALDH1 were present in a minority of cases (4.9% and 35.3%, respectively). Concerning to the observed associations between CD44 expression and clinicopathological variables, we verified that this protein was only significantly associated with the breast cancer histological type, being present in all carcinomas, but with a lower positivity in the solid type. Interestingly, this finding is in accordance with a previous study performed by Madrazo et al. (2009) in canine mammary carcinomas, which showed that CD44 expression is frequently reduced in this tumour type (Madrazo et al., 2009). Although we did not observe a significant association between CD44 expression and the histological grade, there was trend for a reduction in its positivity from differentiated (grade I) to undifferentiated (grade III) carcinomas. Madrazo et al. (2009) also described a significant reduction from grade I to grade II carcinomas; however, all grade III carcinomas evaluated by these investigators showed CD44 expression (Madrazo et al., 2009). Although the differences observed between our studies, we believe that these might be due to the small number of grade

III cases used in their evaluation (n=4). In this study, no association was also found between CD44 expression and patient survival, with similar survival rates observed for dogs harbouring CD44 positive or negative carcinomas. Indeed, Paltian et al. (2009) showed that up-regulation of CD44 appears to be associated with a benign biological behaviour of canine mammary tumours; however, no survival studies were performed in this study (Paltian et al., 2009).

It is known that CD44 exists as distinct isoforms, and several splice variants have been studied as markers of disease and progression. In human breast cancer, several studies have associated the expression of this cell surface protein with stem-like characteristics (Park et al., 2010); however, the role of this protein is still controversial, given the discrepancies in human research literature, which could be a result of the use of distinct antibodies and immunostaining conditions, or reflect differences in cohort composition and number (Kim et al., 2011). Several human breast cancer studies demonstrated that CD44 was significantly expressed in basal-like tumours and aggressive basal-like cell lines (Honeth et al., 2008, Ricardo et al., 2011) and that CD44 positive cells showed a mesenchymal stem cell-like profile, enriched for genes involved in cell motility, proliferation and angiogenesis (Shipitsin et al., 2007). On the other hand, there are studies correlating CD44 expression with better survival and prognosis in several different types of breast cancer, demonstrating that its expression was inversely associated with lymph node metastasis (Gong et al., 2005, Giatromanolaki et al., 2011). It is noteworthy that the largest and most comprehensive studies agree that there is a positive correlation between disease free survival and CD44 expression in human breast cancer (Sleeman and Cremers, 2007, Kim et al., 2011).

Concerning CD24, and to the best of our knowledge, this is the first study evaluating its immunohistochemical expression in canine mammary carcinomas. However, in our study, we did not find any association between CD24 expression and clinicopathological parameters, which might be due to the presence of few positive cases. Fogel et al. (1999) demonstrated for the first time that CD24 could serve as a marker for human breast cancer and that its expression could play an important role in the metastatic process, facilitating the interaction of tumour cells with platelets or vascular endothelial cells (Fogel et al., 1999). Subsequent studies have reported membranous and cytoplasmic CD24 expression as a prognostic indicator of poor survival in breast cancer (Kristiansen et al., 2003, Sleeman and Cremers, 2007, Mylona et al., 2008, Athanassiadou et al., 2009). Kim et al. (2011) verified that CD24 expression was associated with worse prognosis only in hormonal receptor positive breast carcinomas, suggesting that its role in prognosis was related to hormonal receptor status (Kim et al., 2011). Other studies did not confirm these findings and the significance of CD24 expression remains controversial (Abraham et al., 2005, Li et

al., 2010, Ricardo et al., 2011). These contradictory results could, at least partially, be explained by the distinct evaluation systems used to classify CD24 immunohistochemical results, which certainly affects the results concerning both the identification and the prognostic value of this marker (submitted for publication)..

The expression of ALDH1 was observed in 35.3% (36/102) of cases, which is a relatively high percentage, given the low percentage values described in human studies. Ginestier et al. (2007) only found 0.4% (2/481) positive cases (Ginestier et al., 2007), while our group described 7.1% (33/464) (Ricardo et al., 2011) and 13.3% (8/60) positive cases in large series of human breast carcinomas (submitted for publication)..

In this study, we described an association between ALDH1 expression and histological type, with ALDH1 frequently present in tubulopapillary carcinomas and complex carcinomas. Similarly, our group also found ALDH1 expression in papillary carcinoma subtype of a series of human breast carcinomas of special type (submitted for publication).. Interestingly, in our study, its expression was inversely associated with lymphovascular invasion and there was a significant positive association with higher disease free survival and overall survival rates, which is in contrast with human breast cancer studies, where ALDH1 expression is significantly associated with poor prognostic features (Ginestier et al., 2007, Croker et al., 2009, Morimoto et al., 2009, Deng et al., 2010). Although the present study does not confirm ALDH1 marker as a prognostic marker in this animal model, Michishita et al. (2012) has recently demonstrated that $ALDH^{high}$ canine cancer mammary cells are highly tumourigenic. Yet, these cells were sorted using Aldefluor assay, which is not specific for ALDH1A1 activity. Other ALDH isoenzymes, such as ALDH2, are also detected, which opens new possibilities regarding other ALDH families (Moreb et al., 2012).

In our study, we also assessed ALDH1 stromal expression, given that this protein is not restricted to tumour epithelial cells, being also noted in stromal cells (Resetkova et al., 2010). In addition, Resetkova and co-workers (2010) recently described that a high degree of stromal expression was significantly associated with best disease-free survival (Resetkova et al., 2010). Although we have not found any significant associations between its stromal expression and clinicopathological variables or survival, dogs harbouring high to moderate stromal positive carcinomas showed a better disease free survival rates. ALDH1 is a major enzyme involved in the synthesis of retinoic acid, which shows antiproliferative activity on breast cancer cells (Yoshida et al., 1992, Yang et al., 2002). This study performed in human breast cancer tissues suggests that the tumour microenvironment may be as important as tumour cells in determining prognosis. In addition, they did not find an association between epithelial ALDH1 expression and overall or

disease-free survival rates, which illustrates the shortcomings of cell line-based studies and the need to validate the data on tissue studies (Resetkova et al., 2010).

It was recently shown that ALDH1 breast CSC marker can further divide the $CD44^{+}/CD24^{-/low}$ into fractions that are highly tumourigenic, with $CD44^{+}/CD24^{-/low}/ALDH1^{+}$ capable of generating tumours from only 20 cells (Ginestier et al., 2007, Croker et al., 2009). Based on recent evidence, that support the thought that CD44 and CD24 cell surface markers in combination with ALDH1 activity is the most accurate method to identify and isolate CSC-like cells within breast cancer populations (Ginestier et al., 2007), we have analysed $CD44^{+}CD24^{-/low}ALDH^{+}$ expression. Regarding to the expression of this phenotype, we observed that it was only associated with the histological type. Interestingly, the carcinomas that are usually associated with poor prognosis, such as solid carcinomas, carcinosarcomas and anaplastic carcinomas almost did not express this phenotype. No association was observed regarding patient survival, similarly to CD44 or CD24.

Most human breast cancer studies have evaluated CSC markers expression in invasive ductal carcinomas not otherwise specified (IDC-NOS) (Honeth et al., 2008, Ali et al., 2011), with special subtypes usually ignored. As its human counterpart, canine mammary tumours constitute a highly heterogeneous group in terms of morphology and biologic behaviour, and our series reflects this heterogeneity, by including different histological subtypes. This allowed us to demonstrate that CSC markers expression is not homogeneous amongst histological types, similarly to recent findings in human breast cancer (submitted for publication). The fact that our series includes a considerable number of complex carcinomas, which are associated with better prognosis and associated with frequent CSC markers expression might bias our results. Thus, these differences must be considered in future studies investigating the clinical and pathological relevance of CSC markers both in human and in canine mammary cancer.

CONCLUSIONS

Canine mammary cancer constitutes a frequent disease in female dogs, which can ultimately progress to metastasis and death. We hope that our present study would contribute in some way in the understanding of this complex disease. Considering our initial aims and the data presented and discussed herein, we stress the following main conclusions:

- The canine mammary tumour cell lines characterised in the present study (CMT1-m, CMT-2p and CMT-3p) might represent a glimpse of the heterogeneous cell populations found in canine mammary cancer tumours, as they display distinct phenotypical and functional characteristics, namely in their invasive capacity. We believe that these cells will certainly constitute a valuable tool in future canine mammary biopathological research.

- Based on distinct methodologies, we were able to characterise the CSC phenotype of the cell lines under study, revealing different expression patterns: CMT1-m and CMT-2p cell lines showed an increased expression of human mammary stem-cell related markers, by using western blot and immunofluorescence; on the other hand, flow cytometry showed that CMT-2p and CMT-3p were highly enriched for the human $CD44^{+}/CD24^{low}$ breast CSC phenotype.

- Based on its expression pattern, CMT-3p cells seem the ones harbouring a more aggressive *in vitro* phenotype. These cells showed a more mesenchymal phenotype and harboured high levels of cells with the CSC phenotype $CD44^{+}CD24^{-/low}$ and ALDH1 activity, which might explain the high proliferation and invasion capacity of this specific cell line.

- All the cell lines showed capacity to grow in anchorage independent conditions as spheroids, although CMT-3p was the one presenting the higher efficiency. According to our results, no relation seems to exist between the expression of human stem cell-related markers, such as α6- and β4-integrins, and the capacity of these cell lines to grow as mammospheres. Yet, it seems to occur an association between the ALDH1 activity and $CD44^{+}/CD24^{low}$ phenotype and the capacity to generate mammospheres.

- Epithelial mesenchymal transition, which has been implicated as an important event for cancer invasion and metastasis, was not necessarily associated with stem-cell traits in our *in vitro* model, namely in CMT-2p cells.

- By analysing the expression of CSC markers in a series of canine mammary carcinomas, we have observed that their expression differs across distinct histological types. CD44 was

frequently expressed among primary carcinomas, being seldom found in solid carcinoma subtype. CD24 was rarely found in canine mammary carcinomas, with no statistical differences observed, when compared to clinicopathological parameters. Canine tumours exhibited a relatively high percentage of ALDH1 positivity, given the low percentage values described in human studies. ALDH1 stromal expression showed no association with the evaluated parameters.

- Epithelial ALDH1 neoplastic expression was significantly associated with the absence of vascular invasion and to better survival rates. The results obtained in canine tissues are in contrast to most human studies, especially the ones using *in vitro* models. These results eventually point out the weakness of cell line-based studies and the need to validate *in vitro* data in tissue studies.

- Our results further consolidate the stem cell theory in this animal model; however, *in vivo* models are mandatory and the expression of CSC markers in canine mammary tumours requires additional studies in order to validate the present findings.

REFERENCES

Ablett MP, Singh JK, Clarke RB (2012) Stem cells in breast tumours: Are they ready for the clinic? European Journal of Cancer.

Abraham BK, Fritz P, McClellan M, Hauptvogel P, Athelogou M, Brauch H (2005) Prevalence of CD44+/CD24-/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. Clin Cancer Res 11:1154-1159.

Agiostratidou G, Hulit J, Phillips GR, Hazan RB (2007) Differential cadherin expression: potential markers for epithelial to mesenchymal transformation during tumor progression. J Mammary Gland Biol Neoplasia 12:127-133.

Aigner S, Sthoeger ZM, Fogel M, Weber E, Zarn J, Ruppert M, Zeller Y, Vestweber D, Stahel R, Sammar M, Altevogt P (1997) CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. Blood 89:3385-3395.

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100:3983-3988.

Ali HR, Dawson SJ, Blows FM, Provenzano E, Pharoah PD, Caldas C (2011) Cancer stem cell markers in breast cancer: pathological, clinical and prognostic significance. Breast Cancer Res 13:R118.

Aresu L, Giantin M, Morello E, Vascellari M, Castagnaro M, Lopparelli R, Zancanella V, Granato A, Garbisa S, Arico A, Bradaschia A, Mutinelli F, Dacasto M (2011) Matrix metalloproteinases and their inhibitors in canine mammary tumors. BMC Vet Res 7:33.

Athanassiadou P, Grapsa D, Gonidi M, Athanassiadou AM, Tsipis A, Patsouris E (2009) CD24 expression has a prognostic impact in breast carcinoma. Pathol Res Pract 205:524-533.

Bao B, Wang Z, Ali S, Kong D, Li Y, Ahmad A, Banerjee S, Azmi AS, Miele L, Sarkar FH (2011) Notch-1 induces epithelial-mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells. Cancer Lett 307:26-36.

Barczyk M, Carracedo S, Gullberg D (2010) Integrins. Cell Tissue Res 339:269-280.

Baumann P, Cremers N, Kroese F, Orend G, Chiquet-Ehrismann R, Uede T, Yagita H, Sleeman JP (2005) CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. Cancer Res 65:10783-10793.

Beachy PA, Karhadkar SS, Berman DM (2004) Tissue repair and stem cell renewal in carcinogenesis. Nature 432:324-331.

Benhaj K, Gur B, Bozkurt B, Yulug I, Akcali C, Ozturk M (2005) Expression profiling of Wnt pathway genes in breast cancer. Breast Cancer Research 7:P4.18.

Bhat-Nakshatri P, Appaiah H, Ballas C, Pick-Franke P, Goulet R, Jr., Badve S, Srour EF, Nakshatri H (2010) SLUG/SNAI2 and tumor necrosis factor generate breast cells with CD44+/CD24- phenotype. BMC Cancer 10:411.

Blacking TM, Waterfall M, Argyle DJ (2011) CD44 is associated with proliferation, rather than a specific cancer stem cell population, in cultured canine cancer cells. Veterinary Immunology and Immunopathology 141:46-57.

Brennan KR, Brown AM (2004) Wnt proteins in mammary development and cancer. J Mammary Gland Biol Neoplasia 9:119-131.

Brown RL, Reinke LM, Damerow MS, Perez D, Chodosh LA, Yang J, Cheng C (2011) CD44 splice isoform switching in human and mouse epithelium is essential for epithelial-mesenchymal transition and breast cancer progression. J Clin Invest 121:1064-1074.

Burstein HJ (2005) The distinctive nature of HER2-positive breast cancers. N Engl J Med 353:1652-1654.

Campbell LL, Polyak K (2007) Breast tumor heterogeneity: cancer stem cells or clonal evolution? Cell Cycle 6:2332-2338.

Carroll DK, Carroll JS, Leong CO, Cheng F, Brown M, Mills AA, Brugge JS, Ellisen LW (2006) p63 regulates an adhesion programme and cell survival in epithelial cells. Nat Cell Biol 8:551-561.

Charafe-Jauffret E, Ginestier C, Birnbaum D (2009a) Breast cancer stem cells: tools and models to rely on. BMC Cancer 9:202.

Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J, Brown M, Viens P, Xerri L, Bertucci F, Stassi G, Dontu G, Birnbaum D, Wicha MS (2009b) Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. Cancer Res 69:1302-1313.

Charafe-Jauffret E, Monville F, Ginestier C, Dontu G, Birnbaum D, Wicha MS (2008) Cancer stem cells in breast: current opinion and future challenges. Pathobiology 75:75-84.

Cocola C, Anastasi P, Astigiano S, Piscitelli E, Pelucchi P, Vilardo L, Bertoli G, Beccaglia M, Veronesi MC, Sanzone S, Barbieri O, Reinbold RA, Luvoni GC, Zucchi I (2009) Isolation of canine mammary cells with stem cell properties and tumour-initiating potential. Reprod Domest Anim 44 Suppl 2:214-217.

Constantinidou A, Jones RL, Reis-Filho JS (2010) Beyond triple-negative breast cancer: the need to define new subtypes. Expert Rev Anticancer Ther 10:1197-1213.

Creighton CJ, Chang JC, Rosen JM (2010) Epithelial-mesenchymal transition (EMT) in tumor-initiating cells and its clinical implications in breast cancer. J Mammary Gland Biol Neoplasia 15:253-260.

Croker AK, Goodale D, Chu J, Postenka C, Hedley BD, Hess DA, Allan AL (2009) High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. J Cell Mol Med 13:2236-2252.

Deng S, Yang X, Lassus H, Liang S, Kaur S, Ye Q, Li C, Wang LP, Roby KF, Orsulic S, Connolly DC, Zhang Y, Montone K, Butzow R, Coukos G, Zhang L (2010) Distinct expression levels and patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human epithelial cancers. PLoS One 5:e10277.

Destexhe E, Lespagnard L, Degeyter M, Heymann R, Coignoul F (1993) Immunohistochemical Identification of Myoepithelial, Epithelial, and Connective Tissue Cells in Canine Mammary Tumors. Veterinary Pathology Online 30:146-154.

Dick JE (2003) Breast cancer stem cells revealed. Proc Natl Acad Sci U S A 100:3547-3549.

Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17:1253-1270.

Dontu G, Jackson K, McNicholas E, Kawamura M, Abdallah W, Wicha M (2004) Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast Cancer Res 6:R605 - R615.

Farnie G, Clarke R (2007) Mammary Stem Cells and Breast Cancer—Role of Notch Signalling. Stem Cell Reviews and Reports 3:169-175.

Ferletta M, Grawe J, Hellmen E (2011) Canine mammary tumors contain cancer stem-like cells and form spheroids with an embryonic stem cell signature. Int J Dev Biol 55:791-799.

Fogel M, Friederichs J, Zeller Y, Husar M, Smirnov A, Roitman L, Altevogt P, Sthoeger ZM (1999) CD24 is a marker for human breast carcinoma. Cancer Lett 143:87-94.

Foulkes WD (2004) BRCA1 functions as a breast stem cell regulator. J Med Genet 41:1-5.

Gadalla SE, Alexandraki A, Lindstrom MS, Nister M, Ericsson C (2011) Uncoupling of the ERalpha regulated morphological phenotype from the cancer stem cell phenotype in human breast cancer cell lines. Biochem Biophys Res Commun 405:581-587.

Gama A (2011) A novel myoepithelial cell marker in canine mammary tissue. Vet J.

Gama A, Alves A, Gartner F, Schmitt F (2003) p63: a novel myoepithelial cell marker in canine mammary tissues. Vet Pathol 40:412-420.

Gama A, Alves A, Schmitt F (2008a) Identification of molecular phenotypes in canine mammary carcinomas with clinical implications: application of the human classification. Virchows Arch 453:123-132.

Gama A, Alves A, Schmitt F (2010) Expression and prognostic significance of CK19 in canine malignant mammary tumours. Vet J 184:45-51.

Gama A, Paredes J, Albergaria A, Gartner F, Schmitt F (2004) P-cadherin expression in canine mammary tissues. J Comp Pathol 130:13-20.

Gama A, Paredes J, Gartner F, Alves A, Schmitt F (2008b) Expression of E-cadherin, P-cadherin and beta-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival. Veterinary Journal 177:45-53.

Gamallo C, Palacios J, Suarez A, Pizarro A, Navarro P, Quintanilla M, Cano A (1993) Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. Am J Pathol 142:987-993.

Giatromanolaki A, Sivridis E, Fiska A, Koukourakis MI (2011) The CD44+/CD24- phenotype relates to 'triple-negative' state and unfavorable prognosis in breast cancer patients. Med Oncol 28:745-752.

Gilcrease MZ (2007) Integrin signaling in epithelial cells. Cancer Lett 247:1-25.

Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1:555-567.

Glazer RI, Xiaoyang W, Hongyan Y, Yuzhi Y (2007) Mammary stem and progenitor cell regulation. Cancer Biomarkers 3:171-181.

Goldschmidt M, Pena L, Rasotto R, Zappulli V (2011) Classification and grading of canine mammary tumors. Vet Pathol 48:117-131.

Gong Y, Sun X, Huo L, Wiley EL, Rao MS (2005) Expression of cell adhesion molecules, CD44s and E-cadherin, and microvessel density in invasive micropapillary carcinoma of the breast. Histopathology 46:24-30.

Griffey SM, Madewell BR, Dairkee SH, Hunt JE, Naydan DK, Higgins RJ (1993) Immunohistochemical reactivity of basal and luminal epithelium-specific cytokeratin antibodies within normal and neoplastic canine mammary glands. Vet Pathol 30:155-161.

Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, Gomis RR, Manova-Todorova K, Massague J (2007) Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. Nature 446:765-770.

Gusterson BA, Ross DT, Heath VJ, Stein T (2005) Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. Breast Cancer Res 7:143-148.

Harrell PC, McCawley LJ, Fingleton B, McIntyre JO, Matrisian LM (2004) Proliferative effects of apical, but not basal, matrix metalloproteinase-7 activity in polarized MDCK cells. Experimental Cell Research 303:308-320.

Hayashida T, Jinno H, Kitagawa Y, Kitajima M (2011) Cooperation of cancer stem cell properties and epithelial-mesenchymal transition in the establishment of breast cancer metastasis. J Oncol 2011:591427.

Hazan RB, Kang L, Whooley BP, Borgen PI (1997) N-cadherin promotes adhesion between invasive breast cancer cells and the stroma. Cell Adhes Commun 4:399-411.

Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA (2000) Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol 148:779-790.

Hill RP (2006) Identifying cancer stem cells in solid tumors: case not proven. Cancer Res 66:1891-1895; discussion 1890.

Honeth G, Bendahl PO, Ringner M, Saal LH, Gruvberger-Saal SK, Lovgren K, Grabau D, Ferno M, Borg A, Hegardt C (2008) The CD44+/CD24- phenotype is enriched in basal-like breast tumors. Breast Cancer Res 10:R53.

Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110:673-687.

Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119:1420-1428.

Kasper M, Jaks V, Fiaschi M, Toftgard R (2009) Hedgehog signalling in breast cancer. Carcinogenesis 30:903-911.

Kim HJ, Kim MJ, Ahn SH, Son BH, Kim SB, Ahn JH, Noh WC, Gong G (2011) Different prognostic significance of CD24 and CD44 expression in breast cancer according to hormone receptor status. Breast 20:78-85.

Kim MJ, Ro JY, Ahn SH, Kim HH, Kim SB, Gong G (2006) Clinicopathologic significance of the basal-like subtype of breast cancer: a comparison with hormone receptor and Her2/neu-overexpressing phenotypes. Hum Pathol 37:1217-1226.

Knudsen KA, Wheelock MJ (2005) Cadherins and the mammary gland. Journal of Cellular Biochemistry 95:488-496.

Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M (2007) Vimentin and epithelial-mesenchymal transition in human breast cancer--observations in vitro and in vivo. Cells Tissues Organs 185:191-203.

Korkaya H, Paulson A, Iovino F, Wicha MS (2008) HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. Oncogene 27:6120-6130.

Kristiansen G, Winzer KJ, Mayordomo E, Bellach J, Schluns K, Denkert C, Dahl E, Pilarsky C, Altevogt P, Guski H, Dietel M (2003) CD24 expression is a new prognostic marker in breast cancer. Clin Cancer Res 9:4906-4913.

Li J, Zhou BP (2011) Activation of beta-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters. BMC Cancer 11:49.

Li W, Liu F, Lei T, Xu X, Liu B, Cui L, Wei J, Guo X, Lang R, Fan Y, Gu F, Tang P, Zhang X, Fu L (2010) The clinicopathological significance of CD44+/CD24-/low and CD24+ tumor cells in invasive micropapillary carcinoma of the breast. Pathol Res Pract 206:828-834.

Lindeman GJ, Visvader JE (2010) Insights into the cell of origin in breast cancer and breast cancer stem cells. Asia Pac J Clin Oncol 6:89-97.

Lindley LE, Briegel KJ (2010) Molecular characterization of TGFbeta-induced epithelial-mesenchymal transition in normal finite lifespan human mammary epithelial cells. Biochem Biophys Res Commun 399:659-664.

Liu S, Dontu G, Wicha M (2005) Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Research 7:86 - 95.

Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, Perou CM (2006) Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol 19:264-271.

Luo M, Fan H, Nagy T, Wei H, Wang C, Liu S, Wicha MS, Guan JL (2009) Mammary epithelial-specific ablation of the focal adhesion kinase suppresses mammary tumorigenesis by affecting mammary cancer stem/progenitor cells. Cancer Res 69:466-474.

Lynch CC, Vargo-Gogola T, Matrisian LM, Fingleton B (2010) Cleavage of E-Cadherin by Matrix Metalloproteinase-7 Promotes Cellular Proliferation in Nontransformed Cell Lines via Activation of RhoA. J Oncol 2010:530745.

Madrazo J, Garcia-Fernandez RA, Garcia-Iglesias MJ, Duran AJ, Espinosa J, Perez-Martinez C (2009) The role of CD44 adhesion factor in canine mammary carcinomas. Vet J 180:371-376.

Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133:704-715.

Matos I, Dufloth R, Alvarenga M, Zeferino LC, Schmitt F (2005) p63, cytokeratin 5, and P-cadherin: three molecular markers to distinguish basal phenotype in breast carcinomas. Virchows Arch 447:688-694.

Mercurio AM, Bachelder RE, Chung J, O'Connor KL, Rabinovitz I, Shaw LM, Tani T (2001a) Integrin laminin receptors and breast carcinoma progression. J Mammary Gland Biol Neoplasia 6:299-309.

Mercurio AM, Rabinovitz I, Shaw LM (2001b) The alpha 6 beta 4 integrin and epithelial cell migration. Curr Opin Cell Biol 13:541-545.

Meyer MJ, Fleming JM, Ali MA, Pesesky MW, Ginsburg E, Vonderhaar BK (2009) Dynamic regulation of CD24 and the invasive, CD44posCD24neg phenotype in breast cancer cell lines. Breast Cancer Res 11:R82.

Michishita M, Akiyoshi R, Suemizu H, Nakagawa T, Sasaki N, Takemitsu H, Arai T, Takahashi K (2012) Aldehyde dehydrogenase activity in cancer stem cells from canine mammary carcinoma cell lines. The Veterinary Journal.

Michishita M, Akiyoshi R, Yoshimura H, Katsumoto T, Ichikawa H, Ohkusu-Tsukada K, Nakagawa T, Sasaki N, Takahashi K (2010) Characterization of spheres derived from canine mammary gland adenocarcinoma cell lines. Res Vet Sci.

Miller SJ, Lavker RM, Sun TT (2005) Interpreting epithelial cancer biology in the context of stem cells: tumor properties and therapeutic implications. Biochim Biophys Acta 1756:25-52.

Misdorp W, Else RW, Hellmén E, Lipscomb TP (1999) Histological Classification of Mammary Tumors of the Dog and the Cat VII:18-23.

Monroe MM, Anderson EC, Clayburgh DR, Wong MH (2011) Cancer stem cells in head and neck squamous cell carcinoma. J Oncol 2011:762-780.

Moreb JS, Ucar D, Han S, Amory JK, Goldstein AS, Ostmark B, Chang LJ (2012) The enzymatic activity of human aldehyde dehydrogenases 1A2 and 2 (ALDH1A2 and ALDH2) is detected by Aldefluor, inhibited by diethylaminobenzaldehyde and has significant effects on cell proliferation and drug resistance. Chem Biol Interact 195:52-60.

Morel AP, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux A (2008) Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One 3:e2888.

Morimoto K, Kim SJ, Tanei T, Shimazu K, Tanji Y, Taguchi T, Tamaki Y, Terada N, Noguchi S (2009) Stem cell marker aldehyde dehydrogenase 1-positive breast cancers are characterized by negative estrogen receptor, positive human epidermal growth factor receptor type 2, and high Ki67 expression. Cancer Sci 100:1062-1068.

Munson L, Moresco A (2007) Comparative pathology of mammary gland cancers in domestic and wild animals. Breast Dis 28:7-21.

Murphy LC, Watson P (2002) Steroid receptors in human breast tumorigenesis and breast cancer progression. Biomed Pharmacother 56:65-77.

Mylona E, Giannopoulou I, Fasomytakis E, Nomikos A, Magkou C, Bakarakos P, Nakopoulou L (2008) The clinicopathologic and prognostic significance of CD44+/CD24(-/low) and CD44-/CD24+ tumor cells in invasive breast carcinomas. Hum Pathol 39:1096-1102.

Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ (1999) N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J Cell Biol 147:631-644.

Ohene-Abuakwa Y, Pignatelli M (2000) Adhesion molecules in cancer biology. In: Cancer Gene Therapy, vol. 465, pp 115-126 New York: Kluwer Academic / Plenum Publ.

Oliveira J, Matos A, Lopes C, Hespanhol V, Bernardes ES, G‰rtner F (2009) CD43 Expression in Canine Malignant Mammary Tumours. Journal of Comparative Pathology 141:302-302.

Paltian V, Alldinger S, Baumgartner W, Wohlsein P (2009) Expression of CD44 in canine mammary tumours. J Comp Pathol 141:237-247.

Pang LY, Cervantes-Arias A, Else RW, Argyle DJ (2011) Canine Mammary Cancer Stem Cells are Radio- and Chemo- Resistant and Exhibit an Epithelial-Mesenchymal Transition Phenotype. Cancers 3:1744-1762.

Pardal R, Clarke MF, Morrison SJ (2003) Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3:895-902.

Paredes J, Albergaria A, Oliveira JT, Jeronimo C, Milanezi F, Schmitt FC (2005) P-cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation. Clinical Cancer Research 11:5869-5877.

Paredes J, Figueiredo J, Albergaria A, Oliveira P, Carvalho J, Ribeiro AS, Caldeira J, Costa AM, Simoes-Correia J, Oliveira MJ, Pinheiro H, Pinho SS, Mateus R, Reis CA, Leite M, Fernandes MS, Schmitt F, Carneiro F, Figueiredo C, Oliveira C, Seruca R (2012) Epithelial E- and P-cadherins: Role and clinical significance in cancer. Biochim Biophys Acta 1826:297-311.

Paredes J, Milanezi F, Viegas L, Amendoeira I, Schmitt F (2002) P-cadherin expression is associated with high-grade ductal carcinoma in situ of the breast. Virchows Archiv 440:16-21.

Park SY, Lee HE, Li H, Shipitsin M, Gelman R, Polyak K (2010) Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. Clin Cancer Res 16:876-887.

Pearce ST, Jordan VC (2004) The biological role of estrogen receptors alpha and beta in cancer. Crit Rev Oncol Hematol 50:3-22.

Peinado H, Olmeda D, Cano A (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 7:415-428.

Pinho SS, Carvalho S, Cabral J, Reis CA, GV§rtner Ft (2012) Canine tumors: a spontaneous animal model of human carcinogenesis. Translational Research 159:165-172.

Polyak K (2007a) Breast cancer stem cells: a case of mistaken identity? Stem Cell Rev 3:107-109.

Polyak K (2007b) Breast cancer: origins and evolution. J Clin Invest 117:3155-3163.

Polyak K, Hu M (2005) Do myoepithelial cells hold the key for breast tumor progression? J Mammary Gland Biol Neoplasia 10:231-247.

Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG (2005) Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 65:5506-5511.

Pontier SM, Muller WJ (2009) Integrins in mammary-stem-cell biology and breast-cancer progression--a role in cancer stem cells? J Cell Sci 122:207-214.

Powell WC, Fingleton B, Wilson CL, Boothby M, Matrisian LM (1999) The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr Biol 9:1441-1447.

Radisky DC, LaBarge MA (2008) Epithelial-mesenchymal transition and the stem cell phenotype. Cell Stem Cell 2:511-512.

Rakha EA, Putti TC, Abd El-Rehim DM, Paish C, Green AR, Powe DG, Lee AH, Robertson JF, Ellis IO (2006) Morphological and immunophenotypic analysis of breast carcinomas with basal and myoepithelial differentiation. J Pathol 208:495-506.

Raouf A, Zhao Y, To K, Stingl J, Delaney A, Barbara M, Iscove N, Jones S, McKinney S, Emerman J, Aparicio S, Marra M, Eaves C (2008) Transcriptome analysis of the normal human mammary cell commitment and differentiation process. Cell Stem Cell 3:109-118.

Raymond K, Faraldo MM, Deugnier MA, Glukhova MA (2012) Integrins in mammary development. Semin Cell Dev Biol.

Reis-Filho JS, Tutt AN (2008) Triple negative tumours: a critical review. Histopathology 52:108-118.

Resetkova E, Reis-Filho JS, Jain RK, Mehta R, Thorat MA, Nakshatri H, Badve S (2010) Prognostic impact of ALDH1 in breast cancer: a story of stem cells and tumor microenvironment. Breast Cancer Res Treat 123:97-108.

Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105-111.

Ricardo S, Vieira AF, Gerhard R, Leitao D, Pinto R, Cameselle-Teijeiro JF, Milanezi F, Schmitt F, Paredes J (2011) Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. J Clin Pathol.

Rodriguez FJ, Lewis-Tuffin LJ, Anastasiadis PZ (2012) E-cadherin's dark side: Possible role in tumor progression. Biochim Biophys Acta 1826:23-31.

Rutteman GR, Misdorp W, Blankenstein MA, van den Brom WE (1988) Oestrogen (ER) and progestin receptors (PR) in mammary tissue of the female dog: different receptor profile in non-malignant and malignant states. Br J Cancer 58:594-599.

Rutteman GR, Withrow SJ, MacEwen EG (2001) Tumors of the mammary gland. Philadelphia WB Saunders Company.

Sassi F, Benazzi C, Castellani G, Sarli G (2010) Molecular-based tumour subtypes of canine mammary carcinomas assessed by immunohistochemistry. BMC Vet Res 6:5.

Shaw LM (1999) Integrin function in breast carcinoma progression. J Mammary Gland Biol Neoplasia 4:367-376.

Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, Goulet R, Jr., Badve S, Nakshatri H (2006) CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. Breast Cancer Res 8:R59.

Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, Nikolskaya T, Serebryiskaya T, Beroukhim R, Hu M, Halushka MK, Sukumar S, Parker LM, Anderson KS, Harris LN, Garber JE, Richardson AL, Schnitt SJ, Nikolsky Y, Gelman RS, Polyak K (2007) Molecular definition of breast tumor heterogeneity. Cancer Cell 11:259-273.

Sleeman JP, Cremers N (2007) New concepts in breast cancer metastasis: tumor initiating cells and the microenvironment. Clin Exp Metastasis 24:707-715.

Sorenmo K (2003) Canine mammary gland tumors. Vet Clin North Am Small Anim Pract 33:573-596.

Sorenmo KU, Rasotto R, Zappulli V, Goldschmidt MH (2011) Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms. Vet Pathol 48:85-97.

Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98:10869-10874.

Stingl J, Caldas C (2007) Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. Nat Rev Cancer 7:791-799.

Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ (2006) Purification and unique properties of mammary epithelial stem cells. Nature 439:993-997.

Strandberg JD, Goodman DG (1974) Animal model of breast cancer. American Journal of Pathology 75(1):225-228.

Suzuki H, Toyota M, Carraway H, Gabrielson E, Ohmura T, Fujikane T, Nishikawa N, Sogabe Y, Nojima M, Sonoda T, Mori M, Hirata K, Imai K, Shinomura Y, Baylin SB, Tokino T (2008) Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. Br J Cancer 98:1147-1156.

Taddei I, Faraldo MM, Teuliere J, Deugnier MA, Thiery JP, Glukhova MA (2003) Integrins in mammary gland development and differentiation of mammary epithelium. J Mammary Gland Biol Neoplasia 8:383-394.

Takebe N, Warren RQ, Ivy SP (2011) Breast cancer growth and metastasis: interplay between cancer stem cells, embryonic signaling pathways and epithelial-to-mesenchymal transition. Breast Cancer Res 13:211.

Trosko JE (2009) Review paper: cancer stem cells and cancer nonstem cells: from adult stem cells or from reprogramming of differentiated somatic cells. Vet Pathol 46:176-193.

Tysnes BB, Bjerkvig R (2007) Cancer initiation and progression: involvement of stem cells and the microenvironment. Biochim Biophys Acta 1775:283-297.

Uyama R, Nakagawa T, Hong SH, Mochizuki M, Nishimura R, Sasaki N (2006) Establishment of four pairs of canine mammary tumour cell lines derived from primary and metastatic origin and their E-cadherin expression. Vet Comp Oncol 4:104-113.

van Staveren WC, Solis DY, Hebrant A, Detours V, Dumont JE, Maenhaut C (2009) Human cancer cell lines: Experimental models for cancer cells in situ? For cancer stem cells? Biochim Biophys Acta 1795:92-103.

Vieira AF, Ricardo S, Ablett MP, Dionísio MR, Mendes N, Albergaria A, Farnie G, Gerhard R, Cameselle-Teijeiro JF, Seruca R, Schmitt F, Clarke RB, Paredes J (2012) P-Cadherin is Co-Expressed with Cd44 and Cd49f and Mediates Stem Cell Properties in Basal-Like Breast Cancer. STEM CELLS N/A-N/A.

Visvader JE (2011) Cells of origin in cancer. Nature 469:314-322.

Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8:755-768.

Weaver VM, Lelievre S, Lakins JN, Chrenek MA, Jones JC, Giancotti F, Werb Z, Bissell MJ (2002) beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell 2:205-216.

Wicha MS, Liu S, Dontu G (2006) Cancer stem cells: an old idea--a paradigm shift. Cancer Res 66:1883-1890; discussion 1895-1886.

Yamaguchi Y (2007) Microenvironmental regulation of estrogen signals in breast cancer. Breast Cancer 14:175-181.

Yang Q, Sakurai T, Kakudo K (2002) Retinoid, retinoic acid receptor beta and breast cancer. Breast Cancer Res Treat 76:167-173.

Yoshida A, Hsu LC, Dave V (1992) Retinal oxidation activity and biological role of human cytosolic aldehyde dehydrogenase. Enzyme 46:239-244.

Yoshida R, Kimura N, Harada Y, Ohuchi N (2001) The loss of E-cadherin, α- and β-catenin expression is associated with metastasis and poor prognosis in invasive breast cancer. International Journal of Oncology 18:513-520.

Zahir N, Lakins JN, Russell A, Ming W, Chatterjee C, Rozenberg GI, Marinkovich MP, Weaver VM (2003) Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors. J Cell Biol 163:1397-1407.

Zhong X, Rescorla FJ (2011) Cell surface adhesion molecules and adhesion-initiated signaling: Understanding of anoikis resistance mechanisms and therapeutic opportunities. Cellular Signalling 24:393-401.