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ORIGINAL ARTICLE

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

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Abstract Similarly to humans, canine mammary cancer represents a heterogeneous group in terms of morphology and biological behaviour. In the present study, we evaluated a series of canine mammary carcinomas based on a new human classification, initially based on gene expression profiling analysis. Similarly to human breast cancer, by using an immunohistochemistry surrogate panel based on five molecular markers [estrogen receptor, human epidermal growth factor receptor 2 (HER2), cytokeratin 5, p63 and P-cadherin], we were able to classify canine mammary carcinomas into four different subtypes: luminal A [estrogen receptor (ER)+/HER2−; 44.8%], luminal B (ER+/HER2+; 13.5%), basal (ER−/HER2− and a basal marker positive; 29.2%) and HER2 overexpressing tumours (ER−/HER2+; 8.3%). Luminal A-type tumours were characterised by lower grade and proliferation rate, whereas basal-type tumours were mostly high grade, high proliferative and positive for cytokeratin 5, p63 and P-cadherin. In addition, as in humans, basal subtype was significantly associated with shorter disease-free and overall survival rates, and we propose canine mammary carcinomas as a suitable natural model for the study of this particular subset of human carcinomas.

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Introduction

Mammary gland tumours are the most commonly occurring neoplasm in the female dog and represent a remarkably heterogeneous group in terms of morphology and biological behaviour [32, 43]. About half of canine mammary tumours are considered malignant, and the identification of reliable prognostic factors is essential in order to estimate the individual risk of unfavourable clinical outcome [29, 54].

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

Human and canine mammary cancer studies based on single molecular markers probably cannot accurately account for the heterogeneity of this disease [39]. Given the large number of cellular events involved in cell growth, differentiation, proliferation, invasion and metastases [4], the investigation of multiple molecular alterations in concert has been assuming great importance due to the introduction of high-throughput technologies [39]. In fact, recent gene expression profiling studies on human breast tumours have identified distinct molecular subtypes of breast carcinomas, which differ in their pathobiology and

clinical outcomes [36, 47, 48]. Sorlie et al. [48] analysed the expression profiles of 115 sporadic breast tumour samples and categorised them into five main groups: luminal A, luminal B, HER2-overexpressing, basal like and normal breast tissue like. Luminal A and B subtypes are based on the expression of estrogen receptor (ER), usually with luminal cytokeratin (CK) expression, whereas the basal-like subtype is characterised by the absence of hormonal receptors and expression of basal cell markers [5, 33].

Given that gene expression profiling is impractical as a routine diagnostic tool, there are immunohistochemistry surrogate panels proposed that can potentially distinguish breast cancer subtypes [27, 33]. In the present study, we sought to identify phenotypical subtypes in canine mammary cancer with possible clinical implications. To accomplish this goal, we have characterised by immunohistochemical analysis 102 canine mammary carcinomas based on the immunohistochemical panel proposed by Matos et al. [27], which involved the evaluation of five molecular markers (ER, HER2, CK5, p63 and P-cadherin).

Materials and methods

Tumour specimens

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

Follow-up data

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

Histological examination

Tumours were diagnosed according to the WHO criteria for canine mammary neoplasms [30]. Clinicopathological variables included in the present study were age, ovariohysterectomy status, contraceptive administration, tumour size, tumour location, tumour histological type and grade, presence of intra-tumoral necrosis, presence of vascular invasion and presence of lymph node metastasis.

Tumours were evaluated for grade in accordance with the Nottingham method for human breast tumours [11] based on the assessment of three morphological features: tubule formation, nuclear pleomorphism and mitotic counts. Each of these features was scored on a scale of 1 to 3 to indicate whether it was present in slight, moderate or marked degree, giving a putative total of three to nine points. Grade was allocated by an arbitrary division of the total points as follows: grade I (well differentiated), 3, 4 or 5 points; grade II (moderately differentiated), 6 or 7 points; and grade III (poorly differentiated), 8 or 9 points.

Immunohistochemistry

Tissue sections were incubated with primary monoclonal antibodies against ER, HER2, CK5, p63, P-cadherin and Ki67. Table 1 summarises the antibodies used and the staining procedures adopted for each antibody. Antigen retrieval was carried out by microwave treatment in a 10 mM citrate buffer, pH 6.0, with the exception of P-cadherin, which was performed with an EDTA buffer, pH 8.0 (Lab Vision, USA) in a boiling bath, during 20 min. For Ki-67, slides were previously incubated with 0.2 mg/mL trypsin (Merck) in phosphate-buffered saline (PBS) for 10 min at 37°C. After cooling (20 min at room temperature), the sections were immersed in 3% hydrogen peroxide

Table 1 Primary monoclonal antibodies and immunostaining protocols used

Antibody	Origin	Clone	Dilution	Pretreatment	Incubation
ER	Novocastra, UK	NCL-LH2	1:40	Microwave	2 h
HER2	Novocastra, UK	NCL-CB11	1:40	Microwave	Overnight
CK5	Neomarkers, USA	XM26	1:25	Microwave	Overnight
P63	Neomarkers, USA	4A4	1:150	Microwave	Overnight
PCAD	BD Transduction, USA	56	1:50	Water bath, 98°C	Overnight
Ki67	Dako, Denmark	Mib1	1:50	Trypsin+microwave	Overnight

(H_2O_2) and distillated water during 30 min to block endogenous peroxidase activity. All slides were then incubated with a blocking serum (Lab Vision) for 10 min and then incubated with the specific antibody. After incubation, slides sections were incubated with biotinylated secondary antibody, followed by streptavidin-conjugated peroxidase (Lab Vision), except for ER and HER2. For these antibodies, a polymeric labelling methodology was used as a detection system (Novolink Polymer Detection System, Novocastra, Newcastle, United Kingdom), following the manufacturer's instructions. Subsequently, the colour was developed with 3,3-diaminobenzidine tetrahydrochloride, and slides were counterstained with Gill's haematoxylin, dehydrated and mounted for evaluation by light microscopy.

Adjacent normal mammary tissues were used as internal positive controls for CK5, p63, P-cadherin (basal and myoepithelial cells) and Ki67. As positive controls, we also used canine uterus sections for ER and a human breast carcinoma with proved amplification (by FISH) and overexpression for HER2. Negative controls were carried out by replacing the primary antibody with PBS.

Evaluation of the immunohistochemical data

Nuclear ER immunoreactivity was considered positive when more than 10% of the neoplastic cells expressed this marker. To evaluate HER2 expression, Herceptest scoring system was applied (0=no membrane staining or <10% of cells stained; 1+=incomplete membrane staining in >10% of cells; 2+=>10% of cells with weak to moderate complete membrane staining; and 3+=strong and complete membrane staining in >10% of cells), with 2+ and 3+ cases considered positive. As for CK5 and p63, a semi-quantitative analysis was performed as follows: 0, <10% positive cells; 1, 10–50% positive cells; and 2, >50% positive cells, with a cytoplasmic (CK5) or nuclear (p63) pattern of cellular distribution. Ki-67 and P-cadherin immunostainings were evaluated as previously described in canine tissues [13, 14]. CK5, p63 and P-cadherin were considered positive when more than 50% of the neoplastic cells expressed each marker.

Statistical analysis

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

Results

Patients and tumour characteristics

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

Immunohistochemistry profiles in canine tumours

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

ER and p63 positive cases showed the characteristic nuclear staining, whereas CK5-positive ones showed a cytoplasmic pattern of expression. HER2 positive tumours showed a membranous staining, and P-cadherin positive tumours showed a cytoplasmic and/or membranous immu-

Table 2 Immunohistochemical results in the present study

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

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

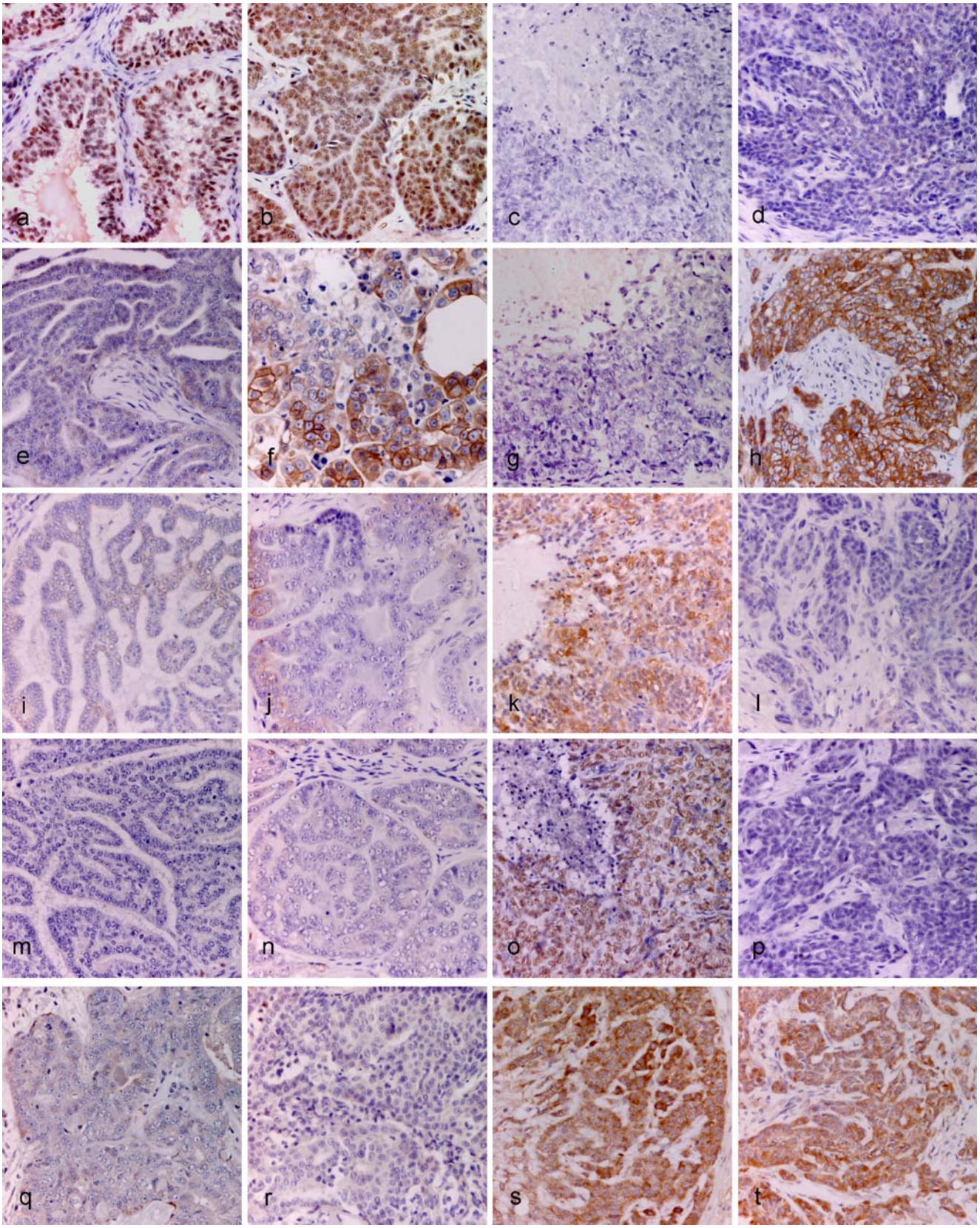

Fig. 1 Immunohistochemical expression of the different proteins studied by IHC in canine mammary carcinomas. **a**–**d** ER staining, **e**–**h** HER2 staining, **i**–**l** CK5 staining, **m**–**p** p63 staining, **q**–**t** P-cadherin staining. Each column represents a distinct molecular subtype. From *left* to *right*, each column represents luminal A, luminal B, basal and HER2 overexpressing subtypes. (Original magnification ×400)

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

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

nostaining. We observed that 58.3% of canine mammary carcinomas in our series were ER positive, whereas 21% were HER2 positive (2+ and 3+). A positive basal cell marker expression was present in 32.4% tumours for both CK5 and p63 and in 42.8% tumours for P-cadherin.

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

Table 4 Association between tumour subtypes and clinicopathological characteristics

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

[a] Lymph nodes were available in 49 cases.

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

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

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

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

With regard to proliferation indices, luminal A tumours showed lower median mitotic and Ki67 labelling indices ($P=0.001$ and $P<0.0001$, respectively), whereas all other groups were characterised by higher proliferation rates, with basal subtype showing the highest Ki67 index.

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

Discussion

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

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

	Luminal A [*n* (%)]	Luminal B [*n* (%)]	Basal [*n* (%)]	HER2 overexpressing [*n* (%)]	*P*
CK5					
Negative	36 (60%)	8 (13.3%)	11 (18.3%)	5 (8.3%)	0.001
Positive	7 (21.9%)	5 (15.6%)	17 (53.1%)	3 (9.4%)	
P63					
Negative	32 (53.3%)	13 (21.7%)	11 (18.3%)	4 (6.7%)	<0.0001
Positive	11 (34.4%)	0 (0%)	17 (53.1%)	4 (12.5%)	
P-cadherin					
Negative	26 (56.5%)	10 (21.7%)	9 (19.6%)	1 (2.2%)	0.001
Positive	13 (31.7%)	3 (7.3%)	18 (43.9%)	7 (17.1%)	
Basal markers					
All negative	21 (77.8%)	6 (22.2%)	0 (0%)	0 (0%)	<0.0001
One positive	11 (39.3%)	6 (21.4%)	8 (28.6%)	3 (10.7%)	
Two positive	5 (20%)	1 (4%)	15 (60%)	4 (16%)	
All positive	2 (25%)	0 (0%)	5 (62.5%)	1 (12.5%)	
Median mitotic index[a] (Min–Max)	0.44 (0–1.59)	1.0 (0.1–2.99)	0.94 (0.1–2.09)	0.7 (0.3–1.9)	0.001
Median Ki67 index[a] (Min–Max)	17.89 (5.39–56.36)	26.7 (15–44.8)	28.14 (12.10–49.2)	26.4 (22.5–35.86)	<0.0001

[a] Proliferative indices were available in 86 cases.

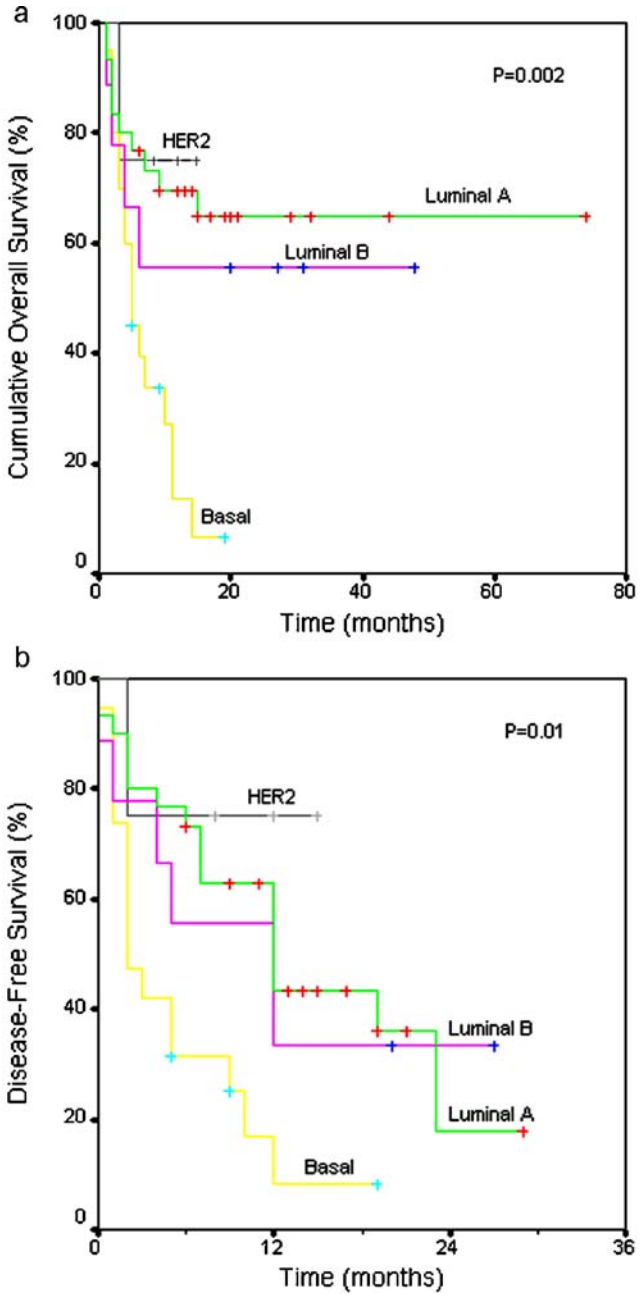

Fig. 2 Kaplan–Meier overall survival (**a**) and disease-free survival (**b**) curves of the different subtype groups

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

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

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

CK5, p63 and P-cadherin are proteins that are expressed early in epithelial differentiation and may contribute to a committed stem cell and/or progenitor phenotype [6, 7, 9, 35]. In this study, we demonstrate that these markers are upregulated in the basal subtype, similarly to the previous results of Matos et al. [27]. In fact, the basal subtype rarely expressed just one basal marker but frequently expressed them simultaneously, which suggests a more undifferentiated profile. HER2-overexpressing subtype was also characterised by an up-regulation of basal markers, confirming some human breast studies, which suggested that HER2-overexpressing tumours should be included in a bona fide basal-like subclass [5, 27]. In contrast, the majority of luminal tumours in our series were simultaneously negative for basal cell markers, with some cases showing basal marker expression, which was also described by some authors who reported tumours co-expressing basal CK and hormone receptors or HER2 [40, 50].

Similarly to human breast cancers, in this study, we further demonstrate the molecular heterogeneity of canine mammary cancer. A "hierarchy or stem cell" model of breast cancer oncogenesis has been proposed to elucidate the observed functional heterogeneity of tumours. In this model, transformation occurs in a stem cell or in a

progenitor "highly proliferating" cell, and expansion proceeds until various maturation stages, depending on the genomic alterations. Specific genetic alterations would lead to distinct cellular transcriptomic programmes, including the change of hormonal receptors and CK expression pattern, characterising distinct subgroups of breast carcinomas [5, 8, 39].

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

In contrast to basal subgroup, luminal and HER2 overexpressing subtypes showed increased survival rates. The fact that luminal tumours were associated with a better prognosis is not surprising since ER positive human breast carcinomas are usually associated with a more favourable clinical outcome. In veterinary pathology, however, the prognostic value of ER in canine mammary cancer is still a matter of debate. Previous studies using biochemical [45] and immunohistochemical [34] methodologies have demonstrated the prognostic value of ER, but others have failed this confirmation [23, 28]. The observed discrepancies between different studies are probably related with sample selection, differences in antibodies, staining procedure and evaluation or sensitivity of the detection system. In our series, luminal tumours were mostly of complex type, which comes in accordance to previous canine studies reporting complex carcinomas as being more likely ER positive [15, 23, 28]. Given that this tumour type is generally associated with a better clinical outcome, its high proportion in luminal subtype groups is probably in part responsible for their favourable prognosis.

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

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

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Conflict of interest statement We declare that we have no conflict of interest.

References

1. Abd El-Rehim DM, Pinder SE, Paish CE, Bell J, Blamey RW, Robertson JF, Nicholson RI, Ellis IO (2004) Expression of luminal and basal cytokeratins in human breast carcinoma. J Pathol 203:661–671
2. Ahern TE, Bird RC, Church Bird AE, Wolfe LG (1996) Expression of the oncogene c-*erb*B-2 in canine mammary cancers and tumor-derived cell lines. Am J Vet Res 57:693–696
3. Banerjee S, Reis-Filho JS, Ashley S, Steele D, Ashworth A, Lakhani SR, Smith IE (2006) Basal-like breast carcinomas: clinical outcome and response to chemotherapy. J Clin Pathol 59:729–735
4. Beckmann MW, Niederacher D, Schnürch HG, Gusterson BA, Bender HG (1997) Multistep carcinogenesis of breast cancer and tumour heterogeneity. J Mol Med 75:429–439

5. Birnbaum D, Bertucci F, Ginestier C, Tagett R, Jacquemier J, Charafe-Jauffret E (2004) Basal and luminal breast cancers: basic or luminous? Int J Oncol 25:249–258
6. Boecker W, Buerger H (2003) Evidence of progenitor cells of glandular and myoepithelial cell lineages in the human adult female breast epithelium: a new progenitor (adult stem) cell concept. Cell Prolif 36(Suppl 1):73–84
7. Boecker W, Moll R, Poremba C, Holland R, Van Diest PJ, Dervan P, Burger H, Wai D, Ina Diallo R, Brandt B, Herbst H, Schmidt A, Lerch MM, Buchwallow IB (2002) Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept. Lab Invest 82:737–746
8. Dick JE (2003) Breast cancer stem cells revealed. Proc Natl Acad Sci U S A 100:3547–3549
9. DiRenzo J, Signoretti S, Nakamura N, Rivera-Gonzalez R, Sellers W, Loda M, Brown M (2002) Growth factor requirements and basal phenotype of an immortalized mammary epithelial cell line. Cancer Res 62:89–98
10. Dutra AP, Granja NVM, Schmitt FC, Cassali GD (2004) c-erbB-2 expression and nuclear pleomorphism in canine mammary tumors. Braz J Med Biol Res 37:1673–1681
11. Elston CW, Ellis IO (1998) Assessment of histological grade. In: Elston CW, Ellis IO (eds) Systemic pathology. The breast. 3rd edn. Churchill Livingstone, London, pp 365–384
12. Fulford LG, Reis-Filho JS, Ryder K, Jones C, Gillett CE, Hanby A, Easton D, Lakhani SR (2007) Basal-like grade III invasive ductal carcinoma of the breast: patterns of metastasis and long-term survival. Breast Cancer Res 9:R4
13. Gama A, Paredes J, Albergaria A, Gartner F, Schmitt FC (2004) P-cadherin expression in canine mammary tissues. J Comp Pathol 130:13–20
14. Gama A, Paredes J, Gartner F, Alves A, Schmitt FC (2008) Expression of E-cadherin, P-cadherin and b-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival. Vet J. 177:45–53 doi:10.1016/j.tvjl.2007.05.024
15. Geraldes M, Gärtner F, Schmitt F (2000) Immunohistochemical study of hormonal receptors and cell proliferation in normal canine mammary glands and spontaneous mammary tumours. Vet Rec 146:403–406
16. 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
17. Hsu W-L, Huang H-M, Liao J-W, Wong M-L, Chang S-C (2007) Increased survival in dogs with malignant mammary tumours overexpressing HER-2 protein and detection of a silent single nucleotide polymorphism in the canine HER-2 gene. Vet J. doi:10.1016/j.tvjl.2007.10.013
18. Jumppanen M, Gruvberger-Saal S, Kauraniemi P, Tanner M, Bendahl PO, Lundin M, Krogh M, Kataja P, Borg A, Fernö M, Isola J (2007) Basal-like phenotype is not associated with patient survival in estrogen receptor negative breast cancers. Breast Cancer Res 9:R16
19. Karayannopoulou M, Kaldrymidou E, Constantinidis TC, Dessiris A (2005) Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. J Comp Pathol 133:246–252
20. 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. Human Pathol 37:1217–1226
21. Lee CH, Kim WH, Lim JH, Kang MS, Kim DY, Kweon OK (2004) Mutation and overexpression of p53 as a prognostic factor in canine mammary tumors. J Vet Sci 5:63–69
22. 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 Path 19:264–271
23. Martin de las Mulas J, Millán Y, Dios R (2005) A prospective analysis of immunohistochemically determined estrogen receptor a and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog. Vet Pathol 42:200–212
24. Martin de las Mulas J, Ordás J, Millán Y, Fernández-Soria V, Ramón y Cajal S (2003) Oncogene HER-2 in canine mammary gland carcinomas: an immunohistochemical and chromogenic in situ hybridization study. Breast Cancer Res Treat 80:363–367
25. Matos AJ, Lopes CC, Faustino AM, Carvalheira JG, dos Santos MS, Rutteman GR, Gartner F (2006) MIB-1 labelling indices according to clinico-pathological variables in canine mammary tumours: a multivariate study. Anticancer Res 26:1821–1826
26. Matos AJ, Lopes CC, Faustino AM, Carvalheira JG, Rutteman GR, Gärtner MF (2007) E-cadherin, beta-catenin, invasion and lymph node metastases in canine malignant mammary tumours. APMIS 115:327–334
27. 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
28. Millanta F, Calandrella M, Bari G, Niccolini M, Vannozzi I, Poli A (2005) Comparison of steroid receptor expression in normal, dysplastic, and neoplastic canine and feline mammary tissues. Res Vet Sci 79:225–232
29. Misdorp W (2002) Tumours of the mammary gland. In: Meuten DJ (ed) Tumors in domestic animals. 4th edn. Iowa State Press, Iowa, pp 575–606
30. Misdorp W, Else RW, Hellmén E, Lipscomb TP (1999) Histological classification of mammary tumors of the dog and the cat, Vol VII, 2nd series. Armed Forces Institute of Pathology, American Registry of Pathology, Washington D.C., and the World Health Organization Collaborating Center for Worldwide Reference on Comparative Oncology, pp 1–59
31. Misdorp W, Hart AA (1976) Prognostic factors in canine mammary cancer. J Natl Cancer Inst 56:779–786
32. Nerurkar VR, Chitale AR, Jalnakurpar BV, Naik SN, Lalitha VS (1989) Comparative pathology of canine mammary tumours. J Comp Pathol 101:389–397
33. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, Akslen LA, Ragaz J, Gown AM, Gilks CB, van de Rijn M, Perou CM (2004) Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 10:5367–5374
34. Nieto A, Peña L, Perez-Alenza MD, Sanchez MA, Flores JM, Castaño M (2000) Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance. Vet Pathol 37:239–247
35. Peralta Soler A, Knudsen KA, Salazar H, Han AC, Keshgegian AA (1999) P-cadherin expression in breast carcinoma indicates poor survival. Cancer 86:1263–1272
36. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. Nature 406:747–752
37. Rakha EA, El-Rehim DA, Paish C, Green AR, Lee AH, Robertson JF, Blamey RW, Macmillan D, Ellis IO (2006a) Basal phenotype identifies a poor prognostic subgroup of breast cancer of clinical importance. Eur J Cancer 42:3149–3156
38. Rakha EA, El-Sayed ME, Green AR, Paish EC, Lee AH, Ellis IO (2007) Breast carcinoma with basal differentiation: a proposal for

pathology definition based on basal cytokeratin expression. Histopathology 50:434–438
39. Rakha EA, El-Sayed ME, Reis-Filho J, Ellis IO (2008) Pathobiological aspects of basal-like breast cancer. Breast Cancer Res Treat. doi:10.1007/s10549-008-9952-1
40. Rakha EA, Putti TC, Abd El-Rehim DM, Paish C, Green AR, Powe DG, Lee AH, Robertson JF, Ellis IO (2006b) Morphological and immunophenotypic analysis of breast carcinomas with basal and myoepithelial differentiation. J Pathol 208:495–506
41. Revillion F, Bonnetarre J, Peyrat JP (1998) ErbB-2 oncogene in human breast cancer and its clinical significance. Eur J Cancer 34:791–808
42. Rungsipipat A, Tateyama S, Yamaguchi R, Uchida K, Miyoshi N, Hayashi T (1999) Immunohistochemical analysis of c-yes and c-erbB-2 oncogene products and p53 tumor suppressor protein in canine mammary tumors. J Vet Med Sci 61:27–32
43. Rutteman GR, Withrow SJ, MacEwen EG (2001) Tumors of the mammary gland. In: Withrow SJ, MacEwen BR (eds) Small animal clinical oncology. Saunders, Philadelphia, pp 455–477
44. Sarli G, Sassi F, Brunetti B, Benazzi C (2007) Luminal-like A and B types in canine mammary carcinomas. 25th Annual Meeting ESVP, Munich (Germany), pp. 187
45. Sartin EA, Barnes S, Kwapien RP, Wolfe LG (1992) Estrogen and progesterone receptor status of mammary carcinomas and correlation with clinical outcome in dogs. Am J Vet Res 53: 2196–2200
46. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182
47. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, Rijn MV, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lonning PE, 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
48. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lonning PE, Brown PO, Borresen-Dale AL, Botstein D (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 100:8418–8423
49. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET (2003) Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci U S A 100:10393–10398
50. Tan DS, Marchió C, Jones RL, Savage K, Smith IE, Dowsett M, Reis-Filho JS (2008) Triple negative breast cancer: molecular profiling and prognostic impact in adjuvant anthracycline-treated patients. Breast Cancer Res Treat. 111:27–44 doi:10.1007/s10549-007-9756-8
51. Tang P, Wang J, Bourne P (2008) Molecular classifications of breast carcinoma with similar terminology and different definitions: are they the same? Human Pathol 39:506–513
52. van de Rijn M, Perou CM, Tibshirani R, Haas P, Kallioniemi O, Kononen J, Torhorst J, Sauter G, Zuber M, Kochli OR, Mross F, Dieterich H, Seitz R, Ross D, Botstein D, Brown P (2002) Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. Am J Pathol 161:1991–1996
53. van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH (2002) Gene expression profiling predicts clinical outcome of breast cancer. Nature 415:530–536
54. Zaidan Dagli ML (2008) The search for suitable prognostic markers for canine mammary tumors: a promising outlook. Vet J 177:3–5