Histopathological and immunohistochemical analysis of predictive and prognostic markers in spontaneous canine mammary cancer

Vladimír Tancoš[1], Marcel Kovalik[2], Martin Levkut[3], Martina Bobrovská[4], Petra Kolenčíková[4], Ľubomír Straka[5], Zuzana Ševčíková[3], Ondrej Škor[6], Martina Antošová[7], Lukáš Plank[4], Keith L. Thoday[8]

[1]Pavol Jozef Safarik University and Louis Pasteur University Hospital, Institute of Pathology, Košice, Slovak Republic
[2]Anima Group, s.r.o., Veterinary Hospital Anima Vets, Žilina, Slovak Republic
[3]University of Veterinary Medicine and Pharmacy, Department of Morphological Disciplines, Košice, Slovak Republic
[4]Comenius University Bratislava, Jessenius Faculty of Medicine, Department of Pathological Anatomy, Martin, Slovak Republic
[5]Unilabs Slovensko, s.r.o., Department of Pathological Anatomy, Prešov, Slovak Republic
[6]Laboklin GMBH & CO.KG, Labor für Klinische Diagnostik, Bad Kissingen, Germany
[7]University Hospital Martin, Department of Development and Strategic Investments, Martin, Slovak Republic
[8]The University of Edinburgh, The Royal (Dick) School of Veterinary Studies, Department of Veterinary Clinical Sciences, Easter Bush Veterinary Centre, Roslin, Midlothian, Scotland

Received January 23, 2023
Accepted May 4, 2023

Abstract

The study investigates the interspecies similarities between canine and human mammary cancer in the sense of innovative predictive and prognostic tumour markers. Surgical resection specimens with diagnosed spontaneous primary mammary cancer obtained from 100 female canine patients were included in this study. Expression of carbonic anhydrase IX (CAIX) enzyme and human epidermal growth factor receptor 2 (HER2) expression was evaluated immunohistochemically. The study was completed with investigation of Ki67 expression and proliferation with marker of myogenous differentiation. Histopathological grading was performed using the Nottingham/modified Bloom-Richardson system. As in humans, our analysis of canine mammary cancer has shown that CAIX positivity in tumour cells significantly correlates with higher levels of HER2 immunoreactivity ($P = 0.001$), and increased tumour grade ($P < 0.001$). The percentage of smooth muscle actin (SMA) positive cases was significantly higher ($P = 0.002$) in the group of mammary carcinomas with CAIX positivity compared to the tumours that were negative. Using antibody Ki67 proliferative activity was not significantly different between mammary tumours that were CAIX positive and CAIX negative. Canine mammary gland carcinomas may, therefore, represent valuable animal models for the study of hypoxic signaling pathways involved in mammary carcinogenesis in humans. Further research investigating this possibility is required.

Dog, carbonic anhydrase IX enzyme, human epidermal growth factor receptor 2, marker of myogenous differentiation, cell proliferation - Ki67

Mammary cancer is a heterogeneous oncological disease characterized by variability in histopathological characteristics, in biological behaviour, and in the possibilities of therapeutic management (Bergholtz et al. 2022). Besides the analysis of human epidermal growth factor receptor 2 (HER2) expression and other commonly used predictive and prognostic markers in mammary cancer, the study of hypoxic signalling pathways has gained attention in oncological research because of possible therapeutical implications (Gloyeske et al. 2015; Ahn et al. 2020; Bonacho et al. 2020). In the normal cell, HER2 is a key component of a complex signalling network and plays a critical role in the regulation of tissue development, growth, and differentiation (Sundaresan et al. 1999).

Address for correspondence:
Martin Levkut
Department of Morphological Disciplines
University of Veterinary Medicine and Pharmacy
Komenského 73, Košice, Slovak Republic
E-mail: levkut.dvm@gmail.com
http://actavet.vfu.cz/

In such studies, carbonic anhydrase IX (CAIX) has emerged as one of the most promising biological markers of hypoxia in mammary carcinoma tumour cells (Campos et al. 2022). CAIX expression occurs when tumour growth exceeds vascularization due to hypoxia (Maxwell et al. 1999). However, the association between CAIX expression and the degree of differentiation, the HER2 status, as well as other predictive and prognostic biomarkers has not yet been elucidated.

The expression of Ki67 is strongly associated with tumour cell proliferation and growth. The nuclear protein (pKi67) is an established prognostic and predictive indicator for the assessment of biopsies from patients with cancer (Li et al. 2015). The prognostic value of pKi67 has been investigated in a number of studies as a reliable marker in tumours including those of the mammary glands (de Azambuja et al. 2007; Li et al. 2015; Davey et al. 2021).

Smooth muscle actin (SMA) has long been used as a myoepithelial marker in human mammary pathology diagnosis as a sensitive marker of myogenous differentiation (Zaha 2014). It is known that invasive carcinomas lack the myoepithelial cell layer that normally surrounds benign mammary gland tumours (Yeh and Mies 2008) which is why evaluation of SMA positivity may by useful in the current experiment.

To identify causal molecular events driving carcinogenesis and to determine the association between biomarkers characterizing the tumour cells of mammary cancer, the development of cross-species comparison analysis studies is of immense importance. Of the various non-rodent species available, dogs have received most attention as potential animal model for the study of mammary cancer because of the interspecies similarities in the sense of aetiology and pathogenesis of this common malignancy (Abadie et al. 2018).

The aim of this study was to determine the interspecies similarities between canine and human mammary cancer with respect to the innovative predictive and prognostic tumour markers. Surgical resection specimens obtained from female canine patient with spontaneous mammary tumours were evaluated immunohistochemically for the presence of CAIX in tumour cells. This results of CAIX analysis were subsequently compared to the histopathological degree of differentiation (Ki67), the HER2 status and a SMA proliferation.

Materials and Methods

Ethical approval

This study was approved by the Ethics Committee of the Jessenius Faculty of Medicine in Martin (number EK 79/2020).

Histopathological analysis

Surgical resection specimens with diagnosed spontaneous primary mammary cancer obtained from 100 female canine patients were included in this study.

Excised samples were fixed in 10% neutral buffered formalin and paraffin embedded. Samples were routinely processed, and 5-µm thick sections were stained with haematoxylin-eosin. Tumours were allocated to specific histopathological subtypes in accordance with the World Health Organization (WHO) classifications of mammary tumours (WHO Classification of Tumours Editorial Board 2019). Histopathological grading was performed using the Nottingham/modified Bloom-Richardson system (Meyer et al. 2005). Accordingly, based on the level of tubule formation, nuclear pleomorphism, and mitotic count, the evaluated mammary cancer samples were subsequently allocated to one of three categories of histological grade: well-differentiated (grade 1), less-differentiated (grade 2), or poorly differentiated (grade 3). For analytical purposes, a two-tiered system was used which divided the sample into 'poorly-differentiated' (grade 3) and 'better-differentiated' (grade 1 and grade 2) categories.

Immunohistochemical analysis of samples

For immunohistochemistry, antibodies were used to: carbonic anhydrase IX (CAIX) (DAKO, Santa Clara, USA), human epidermal growth factor receptor 2 (HER2) (DAKO), smooth muscle actin (SMA) (DAKO), and indicator of cell proliferation - Ki67 (DAKO), (Table 1). 'Positivity of CAIX' was defined as membranous staining present in at least 5% of tumour cells. Scoring of HER2 was performed in accordance with the guidelines

suggested by the WHO (WHO Classification of Tumours Editorial Board 2019). Accordingly, a 4-tiered system was used which distinguished the '0 degree', '1+ degree', '2+ degree', and '3+ degree' of HER2 expression. For analytical purposes, depending on the degree of HER2 expression, cases were allocated to one of two HER2 categories: 'positivity of HER2' (2+ and 3+ degrees combined) and 'negativity of HER2' (0, and 1+ degrees combined). 'Positivity of SMA' was defined as cytoplasmic staining in at least 5% of cells present in the tumour stroma.

The proliferation activity of tumour cells was determined using Ki67 immunohistochemistry. Based on the proportion of tumour cells positive for the Ki67 marker, mammary carcinoma cases were allocated into 'low proliferative activity' (Ki67 < 14%) or 'high proliferative activity' (Ki67 ≥ 14%).

Table 1. Monoclonal antibodies used in immunohistochemical analysis of 100 canine mammary carcinomas.

Specificity	Clone	Dilution	Source
CAIX mouse MoAb	CAIX/SAV BA	1:200	Agilent DAKO (Santa Clara, USA)
HER2 mouse MoAb	c-erbB-2 Oncoprotein	1:200	Agilent DAKO (Santa Clara, USA)
SMA mouse MoAb	1A4	Ready to use	Agilent DAKO (Santa Clara, USA)
Ki67 mouse MoAb	MIB-1	Ready to use	Agilent DAKO (Santa Clara, USA)

CAIX - carbonic anhydrase IX; HER2 - human epidermal growth factor receptor 2; SMA - smooth muscle actin; Ki67 - proliferation marker

Statistical analysis

Results obtained from immunohistochemical analysis of CAIX were correlated with results of HER2, SMA, proliferative activity determined by Ki67, and with the degree of tumour differentiation. Statistical analysis was performed using two-sample test of the equality of proportions with the continuity correction. A *P* value below 0.05 was considered significant. For statistical computing, the R Core Team language and environment were used.

Results

The vast majority of cases (87/100, 87.0%) were invasive ductal carcinomas of no specific type. Less commonly encountered special subtypes included the tubular carcinoma subtype (8/100, 8%), and invasive lobular carcinomas (5/100, 5%). Considering the degree of differentiation, 51 mammary carcinomas (51.0%) belonged to the 'better differentiated' category (grade 1 or grade 2) and 49 cases (49.0%) were grade 3 tumours.

Carbonic anhydrase IX positivity (Plate XIV, Fig. 1) was demonstrated in 62 mammary carcinomas (62.0%). In the remaining 38 cases (38.0%), the analysed tumour tissue did not contain 5% of tumour cells positive for CAIX. Regarding HER2 expression, positivity at 2+ or 3+ degrees was detected in the tumour tissue (Plate XIV, Fig. 2) of 43 mammary carcinomas (43.0%). A borderline or negative result (1+ or 0 degrees, respectively) of HER2 immunohistochemistry was shown in 57 cases (57.0%). Smooth muscle actin positivity was demonstrated in 40 mammary carcinomas (40.0%). A high proliferative score (i.e., Ki67 ≥ 14%) was noted (Plate XIV, Fig. 3) in 53 cases (53.0%). A Ki67 score that was lower than 14% was identified in 47 mammary carcinomas (47.0%). The results are summarized in Table 2.

Table 2. Distribution of evaluated markers in the analysed samples of 100 canine mammary carcinomas.

Clinicopathological variables		Number (%)
CAIX	Positive	62 (62.0)
	Negative	38 (38.0)
	2+ or	43 (43.0)
HER2	3+	
	1+ or 0	57 (57.0)
SMA	Positive	40 (40.0)
	Negative	60 (60.0)
Ki67	≥ 14%	53 (53.0)
	< 14%	47 (47.0)
Tumour differentiation	Grade 3	53 (53.0)
	Grade 1 & Grade 2	47 (47.0)

CAIX - carbonic anhydrase IX; HER2 - human epidermal growth factor receptor 2; SMA - smooth muscle actin; Ki67 - proliferation marker

Sixty-two mammary carcinomas showed a positivity to CAIX marker (Table 3). Concurrently, these tumours showed HER2 positivity at 2+ or 3+ degrees in 32 cases (51.6%). However, in 38 mammary carcinomas characterized by the absence of CAIX expression, 11 cases of tumours (28.9%) were positive for HER2 marker at 2+ or 3+ degrees with a significance of $P = 0.001$. The percentage of SMA-positive cases was higher ($P = 0.002$) in the group of mammary carcinomas with CAIX positivity compared to tumours that were negative (30/62; 48.4% vs 10/38; 26.3%). A high proliferative activity using antibody Ki67 (Ki67 ≥ 14%) was found in 29 mammary carcinomas with CAIX positivity (46.8%) and in 28 cases which were negative for this immunohistochemical marker (37.7%). The difference was non-significant ($P = 0.151$).

Carbonic anhydrase IX expression significantly correlated with poor differentiation of the evaluated tumours (Table 3): Forty-eight CAIX-positive cases (77.4%) were mammary carcinomas with a histopathological grade of 3 according to the Nottingham/modified Bloom-Richardson system. By contrast, only five CAIX-negative cases (13.2%) were categorized as poorly differentiated, grade 3 mammary carcinomas, which was significant at $P < 0.001$.

Table 3. Results of carbonic anhydrase IX expression with correlation to other markers in 100 canine mammary carcinomas.

Clinicopathological variables		CAIX (n = 100)		*P* value
		Positive (n = 62)	Negative (n= 38)	
HER2	2+ or 3+	32 (51.6%)	11 (28.9%)	0.001
	1+ or 0	30 (48.4%)	27 (71.1%)	
SMA	Positive	30 (48.4%)	10 (26.3%)	0.002
	Negative	32 (51.6%)	28 (73.7%)	
Ki67	≥ 14%	29 (46.8%)	14 (36.8%)	0.151
	< 14%	33 (53.2%)	24 (63.2%)	
Tumour differentiation	Grade 3	48 (77.4%)	5 (13.2%)	< 0.001
	Grade 1 or Grade 2	14 (22.6%)	33 (86.8%)	

CAIX - carbonic anhydrase IX; HER2 - human epidermal growth factor receptor 2; SMA - smooth muscle actin; Ki67 - proliferation marker; n - number of observations

Discussion

Mammary cancer is a heterogeneous oncological disease characterized by variability in histopathological characteristics, in biological behaviour, and in the possibilities of therapeutic management. Comparative cross-species studies are of immense importance for the purpose of identification of causal molecular events driving carcinogenesis and to determine the association between biomarkers characterizing tumour cells of mammary cancer.

In our study, we determined the expression rate of novel predictive and prognostic markers in tumour tissue of canine mammary gland carcinomas and compared it with the results of the corresponding human mammary cancer.

Inadequate blood supply in the tumour tissue is a result of uncoordinated and inappropriate formation of blood vessels in the rapidly proliferating and growing tissue. The resulting hypoxia is of major clinical importance because it is associated with resistance to the most chemotherapeutical approaches (Ilardi et al. 2014; Pastorek and Pastorekova 2015). Malignancies with histomorphological evidence of hypoxia have been found to be more often poorly differentiated, high-grade tumours (van Kuijk et al. 2016). Because

evaluation of the hypoxic state in malignant tumours has clinical implications, there is an ongoing search for immunohistochemical markers determining this tumour feature (Lock et al. 2013). With respect to this aspect, analysis of the expression level of CAIX has become the most promising marker of tumour cell hypoxia (Winum et al. 2009; Monti et al. 2012). The value of CAIX evaluation is underscored by the evidence of its selective expression in mammary gland tumour tissue and its near absence in physiological, non-neoplastic cells (Mahon et al. 2015).

Several associations with other predictive and prognostic markers present in tumour cells of mammary cancer may be found when analyzing CAIX (Furlejova et al. 2015; Alves et al. 2019; Yan et al. 2020). Generali et al. (2006) demonstrated that CAIX positivity correlates with HER2 overexpression in the tumour tissue of human breast cancer. Amplification of HER2 occurs in nearly 25% of all breast cancer types and enhances its aggressiveness (Wolff et al. 2013).

A positive correlation between a higher degree of CAIX expression and higher levels of HER2 immunoreactivity in tumour tissue of human mammary cancer was also evidenced by results presented by other authors (Bartošová et al. 2002; Span et al. 2003). In our study, we have shown that a significant association between CAIX positivity and higher levels of HER2 overexpression seems to be present also in canine mammary gland carcinoma. These results suggest that dogs share similar pathogenetic mechanisms implicated in the process of mammary carcinogenesis as those that occur in humans. It seems from our data that these are related to amplification and/or other molecular factors leading to HER2 overexpression. Further studies investigating the role of HER2 in the pathogenesis of mammary gland carcinoma of dogs are suggested.

In our study, the proportion of canine mammary carcinoma cases with SMA-positive tumour stroma was higher in the tumour group characterized by CAIX expression than in the CAIX-negative group. The analysis of SMA in canine mammary cancer needs further research in mammary carcinoma subgroups which are HER2 positive. In women, it seems that SMA-rich stroma has been linked to resistance to trastuzumab (Kim et al. 2018; Vathiotis et al. 2021). This opens the possibility of combinational therapy in the future.

Carbonic anhydrase IX may serve as a prognostic factor in human breast cancer patients, as is evidenced by several authors (Chia et al. 2001; Watson et al. 2003; Hussain et al 2007). Brennan et al. (2006) concluded that CAIX expression in breast cancer tumour cells is associated with larger tumour size, higher grade, and overall poor prognosis in premenopausal women. In their study, CAIX positivity was also linked to resistance to chemotherapy (Brennan et al. 2006). Results published by other authors also point to the possible association between CAIX positivity and lower overall survival, poor differentiation, and the presence of necrosis in tumour tissue (Furjelová et al. 2014; van Kuijk et al. 2016). Our results suggest that a similar relationship is present also in spontaneous canine mammary gland carcinoma.

The proportion of cases having grade 3 mammary cancer determined according to the Nottingham/modified Bloom-Richardson system was higher in the group of tumours characterized by CAIX positivity compared to the CAIX-negative tumour group. These results suggest that, as in women, CAIX expression may be a negative prognostic marker in canine mammary cancer patients.

In conclusion, our study has shown that the expression CAIX and other novel predictive and prognostic markers in canine mammary gland carcinomas is comparable to the corresponding human breast cancer. As is the case in humans, CAIX positivity in tumour cells is significantly correlated with higher degree of HER2 immunoreactivity, and higher tumour grade determined by the Nottingham/modified Bloom-Richardson system. Mammary carcinomas CAIX positive were mostly SMA positive. Proliferative activity

failed to play a significant role between mammary tumours for CAIX positive and CAIX negative cases. Canine mammary gland carcinoma, therefore, may represent a valuable animal model for the study of hypoxic signaling pathways. Future studies investigating interspecies similarities between canine and human mammary cancers is recommended as they may be relevant to therapy in both species.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

This study was conducted thanks to the support of the project: Integrative strategy in development of personalized medicine of selected malignant tumors and its impact on quality of life, IMTS: 313011V446 under the Operational Programme Integrated Infrastructure, co-financed by the European Regional Development Fund.

References

Abadie J, Nguyen F, Loussouarn D, Peña L, Gama A, Rieder N, Belousov A, Bemelmans I, Jaillardon L, Ibisch C, Campone M 2018: Canine invasive mammary carcinomas as models of human breast cancer. Part 2: immunophenotypes and prognostic significance. Breast Cancer Res Treat **167**: 459-468

Ahn S, Woo JW, Lee K, Park SY 2020: HER2 status in breast cancer: changes in guidelines and complicating factors for interpretation. J Pathol Transl Med **54**: 34-44

Alves WEFM, Bonatelli M, Dufloth R, Kerr LM, Carrara GFA, Alves da Costa RF, Neto CS, Tiezzi D, da Costa Vieira RA, Pinheiro C 2019: CAIX is a predictor of pathological complete response and is associated with higher survival in locally advanced breast cancer submitted to neoadjuvant chemotherapy. BMC Cancer **19**: 1173

de Azambuja E, Cardoso F, de Castro G, Colozza M, Mano MS, Durbecq V, Sotiriou C, Larsimont D, Piccart-Gebhart MJ, Paesmans M 2007: Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12 155 patients. Brit J Cancer **96**: 1504-1513

Bartošová M, Parkkila S, Pohlodek K, Karttunen TJ, Galbavý Š, Mucha V, Harris AL, Pastorek J, Pastoreková S 2002: Expression of carbonic anhydrase IX in breast is associated with malignant tissues and is related to overexpression of c-erbB2: Carbonic anhydrase IX and c-erbB2 in breast cancer. J Pathol **197**: 314-321

Bergholtz H, Lien T, Lingaas F, Sørlie T 2022: Comparative analysis of the molecular subtype landscape in canine and human mammary gland tumors. J Mammary Gland Biol Neoplasia **27**: 171-183

Bonacho T, Rodrigues F, Liberal J 2020: Immunohistochemistry for diagnosis and prognosis of breast cancer: a review. Biotech Histochem **95**: 71-91

Brennan DJ, Jirstrom K, Kronblad Å, Millikan RC, Landberg G, Duffy MJ, Rydén L, Gallagher WM, O'Brien SL 2006: CA IX is an independent prognostic marker in premenopausal breast cancer patients with one to three positive lymph nodes and a putative marker of radiation resistance. Clin Cancer Res **12**: 6421-6431

Campos NSP, de Souza BS, Silva GCP, Porto VA, Chalbatani GM, Lagreca G, Janji B, Suarez ER 2022: Carbonic Anhydrase IX: A Renewed Target for Cancer Immunotherapy. Cancers **14**: 1392

Chia SK, Wykoff CC, Watson PH, Han C, Leek RD, Pastorek J, Gatter KC, Ratcliffe P, Harris AL 2001: Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. J Clin Oncol **19**: 3660-3668

Davey MG, Hynes SO, Kerin MJ, Miller N, Lowery AJ 2021: Ki-67 as a prognostic biomarker in invasive breast cancer. Cancers (Basel) **13**: 4455

Furjelová M, Kovalská M, Jurková K, Horáček J, Carbolová T, Adamkov M 2014: Carbonic anhydrase IX: A promising diagnostic and prognostic biomarker in breast carcinoma. Acta Histochem **116**: 89-93

Furjelova M, Kovalska M, Jurkova K, Chylikova J, Mestanova V, Adamkov M 2015: Correlation of carbonic anhydrase IX expression with clinico-morphological parameters, hormonal receptor status and HER-2 expression in breast cancer. Neoplasma **62**: 88-97

Generali D, Fox SB, Berruti A, Brizzi MP, Campo L, Bonardi S, Wigfield SM, Bruzzi P, Bersiga A, Allevi G, Milani M, Aguggini S, Dogliotti L, Bottini A, Harris AL 2006: Role of carbonic anhydrase IX expression in prediction of the efficacy and outcome of primary epirubicin/tamoxifen therapy for breast cancer. Endocr Relat Cancer **13**: 921-930

Gloyeske NC, Woodard AH, Elishaev E, Yu J, Clark BZ, Dabbs DJ, Bhargava R 2015: Immunohistochemical profile of breast cancer with respect to estrogen receptor and HER2 status. Appl Immunohistochem Mol Morphol **23**: 202-208

Hussain S, Ganesan R, Reynolds G, Gross L, Stevens A, Pastorek J, Murray PG, Perunovic B, Anwar MS, Billingham L, James ND, Spooner D, Poole CJ, Rea DW, Palmer DH 2007: Hypoxia-regulated carbonic anhydrase IX expression is associated with poor survival in patients with invasive breast cancer. Brit J Cancer **96**: 104-109

Ilardi G, Zambrano N, Merolla F, Siano M, Varricchio S, Vecchione M, Rosa G, Mascolo M, Staibano S 2014: Histopathological determinants of tumor resistance: a special look to the immunohistochemical expression of carbonic anhydrase IX in human cancers. Curr Med Chem **21**: 1569-1582

Kim Sa, You D, Jeong Y, Yu J, Kim S, Nam S, Lee J 2018: TP53 upregulates α-smooth muscle actin expression in tamoxifen-resistant breast cancer cells. Oncol Rep **41**: 1075-1082

Li, LT, Jiang G, Chen Q, Zheng JN 2015: Ki67 is a promising molecular target in the diagnosis of cancer (Review). Molecular Med Rep, **11**: 1566-1572

Lock FE, McDonald PC, Lou Y, Serrano I, Chafe SC, Ostlund C, Aparicio S, Winum J-Y, Supuran CT, Dedhar S 2013: Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. Oncogene **32**: 5210-5219

Mahon B, Pinard M, McKenna R 2015: Targeting carbonic anhydrase IX activity and expression. Molecules **20**: 2323-2348

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ 1999: The tumour suppressor protein VHLtargets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature **399**: 271-275

Meyer JS, Alvarez C, Milikowski C, Olson N, Russo I, Russo J, Glass A, Zehnbauer BA, Lister K, Parwaresch R 2005: Breast carcinoma malignancy grading by Bloom–Richardson system vs proliferation index: reproducibility of grade and advantages of proliferation index. Mod Pathol **18**: 1067-1078

Monti MS, Supuran CT, De Simone CG 2012: Carbonic anhydrase IX as a target for designing novel anticancer drugs. Curr Med Chem **19**: 821-830

Pastorek J, Pastorekova S 2015: Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: From biology to clinical use. Semin Cancer Biol **31**: 52-64

Span PN, Bussink J, Manders P, Beex LVAM, Sweep CGJ 2003: Carbonic anhydrase-9 expression levels and prognosis in human breast cancer: association with treatment outcome. Brit J Cancer **89**: 271-276

Sundaresan S, Penuel E, Sliwkowski MX 1999: The biology of human epidermal growth factor receptor 2. Curr Oncol Rep **1**: 16-22

van Kuijk SJA, Yaromina A, Houben R, Niemans R, Lambin P, Dubois LJ 2016: Prognostic significance of carbonic anhydrase IX expression in cancer patients: A meta-analysis. Front Oncol **6**: 69

Vathiotis IA, Moutafi MK, Divakar P, Aung TN, Qing T, Fernandez A, Yaghoobi V, El-Abed S, Wang Y, Guillaume S, Nuciforo P, Huober J, Di Cosimo S, Kim S-B, Harbeck N, Gomez H, Shafi S, Syrigos KN, Fountzilas G, Sotiriou C, Pusztai L, Warren S, Rimm DL 2021: Alpha-smooth muscle actin expression in the stroma predicts resistance to trastuzumab in patients with early-stage HER2-positive breast cancer. Clin Cancer Res **27**: 6156-6163

Watson P, Chia S, Wykoff C, Han C, Leek RD, Sly WS, Gatter KC, Ratcliffe P, Harris AL 2003: Carbonic anhydrase XII is a marker of good prognosis in invasive breast carcinoma. Brit J Cancer **88**: 1065-1070

Winum J-Y, Scozzafava A, Montero J-L, Supuran CT 2009: Inhibition of carbonic anhydrase IX: A new strategy against cancer. Anticancer Agents Med Chem **9**: 693-702

WHO Classification of Tumours Editorial Board 2019: Breast tumours. International Agency for Research, Lyon (France)

Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF 2013: Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol **31**: 3997-4013

Yan H, Xiao H, Zhu J, Zhang J, Liu Z 2020: Association between the HER2 protein expression level and the efficacy of neoadjuvant chemotherapy in HER2-positive breast cancer. Cancer Manag Res **12**: 12715-12722

Yeh IT, Mies C 2008: Application of immunohistochemistry to breast lesions. Arch Pathol Lab Med **132**: 349-358

Zaha DC 2014: Significance of immunohistochemistry in breast cancer. World J Clin Oncol **5**: 382-392

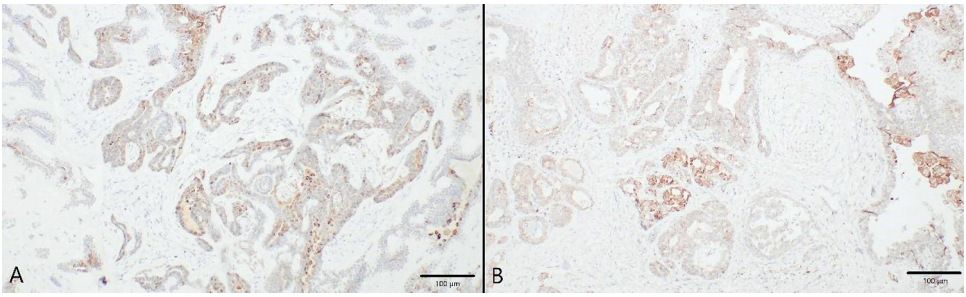

Fig. 1. A, B – Canine mammary carcinomas: carbonic anhydrase IX (CAIX) positivity defined as cytoplasmic staining present in at least 5% of tumour cells (CAIX immunohistochemistry, bar 100 µm)

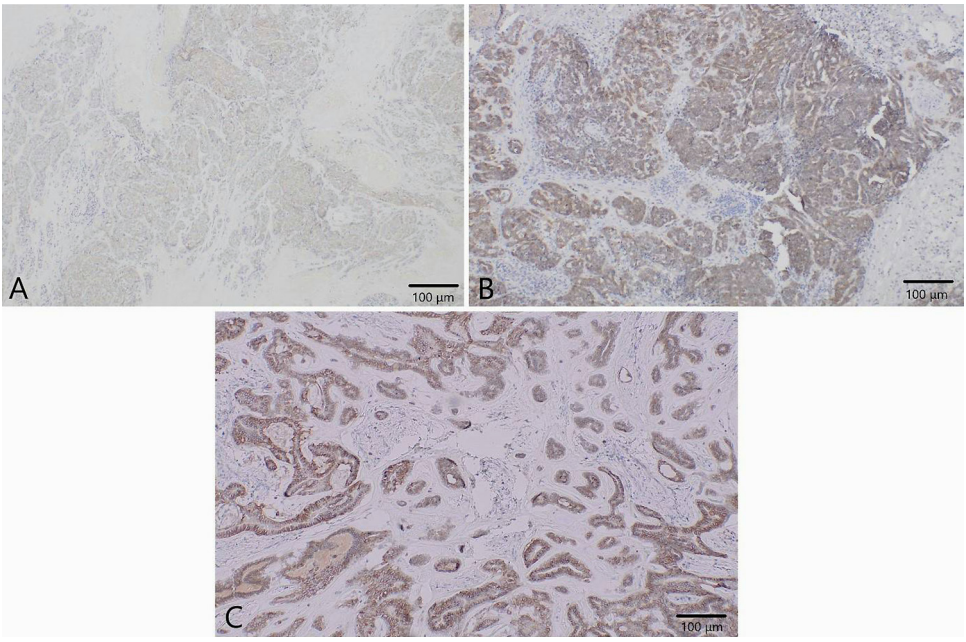

Fig. 2. Canine mammary carcinomas: three degrees of human epidermal growth factor receptor 2 (HER2) positivity: A – degree 1+; B – degree 2+; C – degree 3+; (HER2 imunohistochemistry, bar 100 µm)

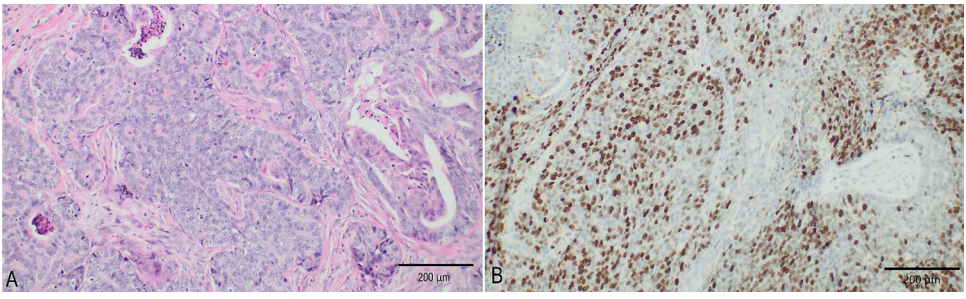

Fig. 3. A - grade 3 canine mammary carcinoma (haematoxylin-eosin), B - corresponding Ki67 positivity in tumour cells (Ki67 immunohistochemistry, bar 200 µm)