Veterinary Record

Confid

Expression of tyrosine kinase receptors PDGFRα, PDGFRβ, VEGFR-2 and CD117 in canine mammary tumours

Journal:	*Veterinary Record*
Manuscript ID	Draft
Article Type:	Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Gattino, Francesca; Universita degli Studi di Torino Scuola di Agraria e Medicina Veterinaria, Scienze Veterinarie Maniscalco, Lorella; Universita degli Studi di Torino Scuola di Agraria e Medicina Veterinaria, Scienze Veterinarie Iussich, Selina; Universita degli Studi di Torino Scuola di Agraria e Medicina Veterinaria, Scienze Veterinarie Biasato, Ilaria; Universita degli Studi di Torino Scuola di Agraria e Medicina Veterinaria, Scienze Veterinarie Martano, Marina; Universita degli Studi di Torino Scuola di Agraria e Medicina Veterinaria, Scienze Veterinarie Morello, Emanuela; Universita degli Studi di Torino Scuola di Agraria e Medicina Veterinaria, Scienze Veterinarie Gola, Cecilia; Universita degli Studi di Torino Scuola di Agraria e Medicina Veterinaria, Scienze Veterinarie Mart&#65533;n de las Mulas, Juana; Universidad de Cordoba, Anatomia y Anatomia Patologica Comparadas Sasaki, Nobuo; Tokyo, Graduate School of Agricultural and Life Sciences Millán, Yolanda; University of Córdoba, Universidad de Cordoba, Anatomia y Anatomia Patologica Comparadas Campus de Rabanales Edificio de Sanidad Animal C&#65533;rdoba, Colorado, ES 14071 34957218681 Buracco, Paolo; Universita degli Studi di Torino Scuola di Agraria e Medicina Veterinaria, Scienze Veterinarie De Maria, Raffaella; Universita degli Studi di Torino Scuola di Agraria e Medicina Veterinaria, Scienze Veterinarie
Abstract:	Canine mammary tumours (CMTs) are one of the most common malignancies in bitches. Platelet-derived growth factor receptors (PDGFR)-Alpha and Beta, vascular endothelial growth factor receptor-2 (VEGFR-2) and CD117 are tyrosine kinase receptors (TKRs) involved in several tumours and represent suitable targets for specific therapy with toceranib phosphate. The purpose of this study was to evaluate the expression of these receptors in the pathogenesis and progression of CMTs. PDGFRα, PDGFRβ, VEGFR-2 and CD117 were expressed in 46/83 (55.4%), 33/83 (39.8%), 46/83 (55.4%) and 32/83 (38.5%) of CMTs, respectively. Immunohistochemical results showed a statistically significant loss of PDGFRα and PDGFRβ expression in simple carcinomas compared to complex/mixed carcinomas. Protein expression by western blot revealed specific bands corresponding to PDGFRα and VEGFR-2 in 3/7 and in 1/7 cell

	lines. Moreover in vitro treatment showed that toceranib phosphate weakly reduced cell proliferation in one canine mammary cell line. These data should be considered for possible therapeutical approaches with specific tyrosine kinase inhibithors (TKI) in this tumor.

SCHOLARONE™
Manuscripts

For Review Only - Confidential

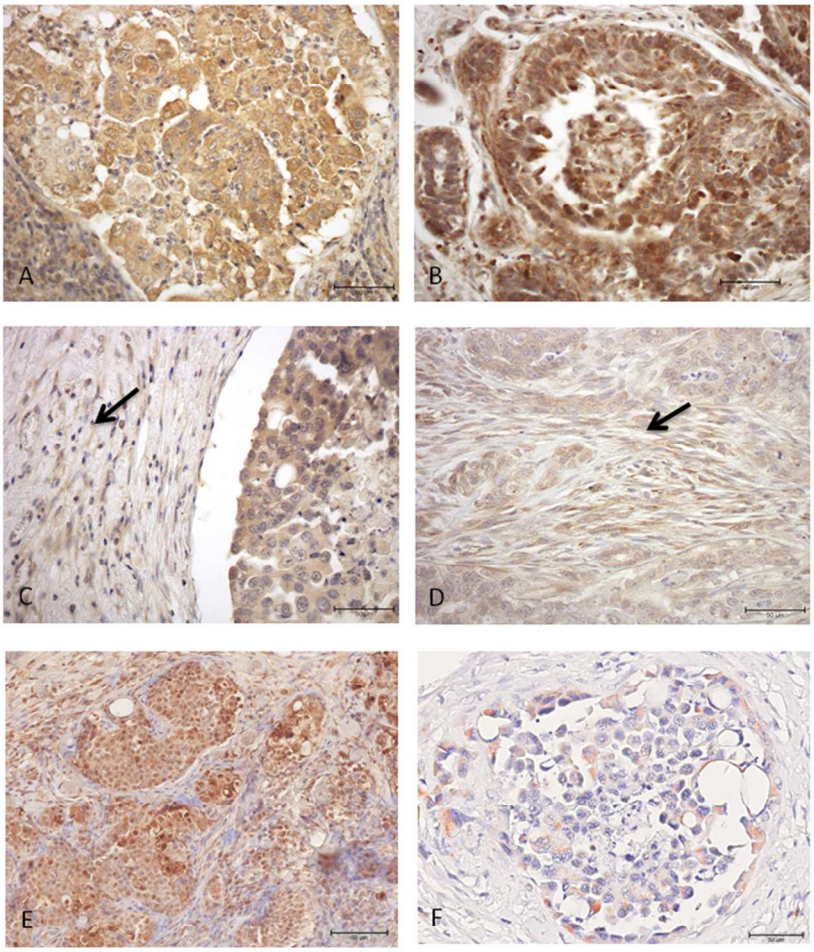


Figure 1
immunohistochemistry
190x275mm (96 x 96 DPI)

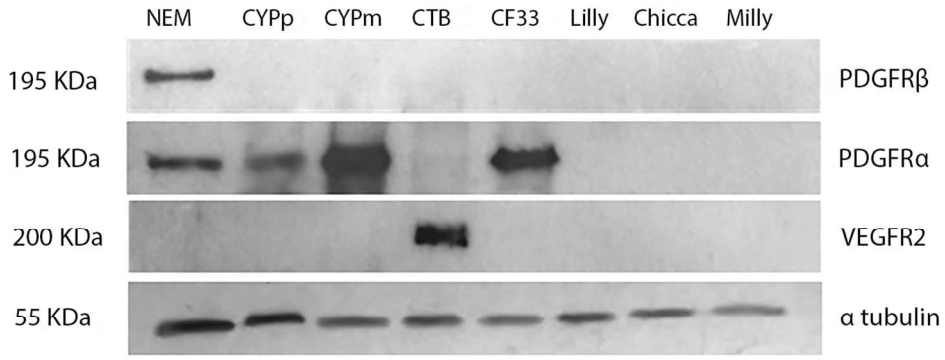


Figure 2
Western blot
119x52mm (300 x 300 DPI)

All: For Review Only

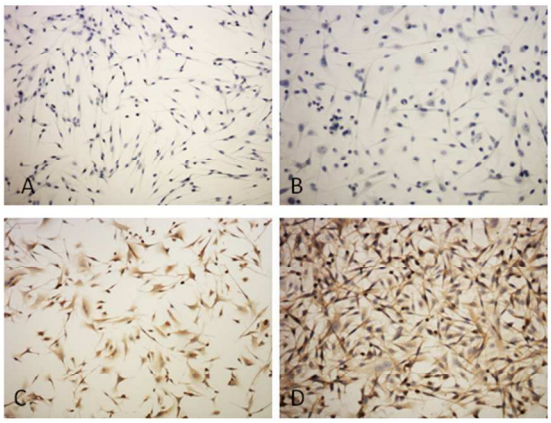
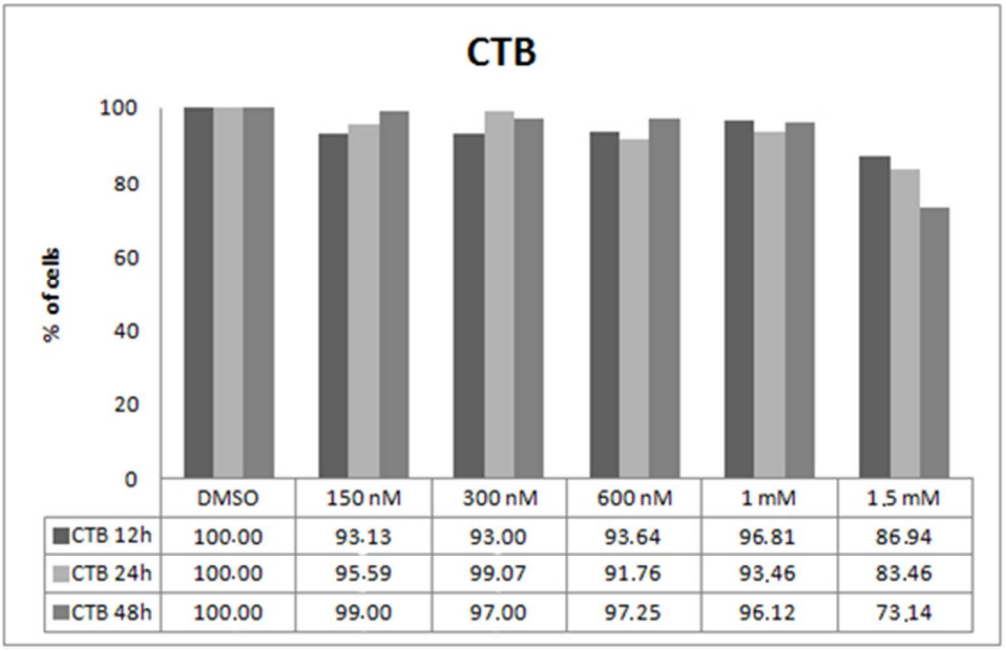


Figure 3
immunocitochemistry
254x190mm (96 x 96 DPI)

Review Only



	DMSO	150 nM	300 nM	600 nM	1 mM	1.5 mM
CTB 12h	100.00	93.13	93.00	93.64	96.81	86.94
CTB 24h	100.00	95.59	99.07	91.76	93.46	83.46
CTB 48h	100.00	99.00	97.00	97.25	96.12	73.14

Figure 4A
proliferating assay
50x32mm (300 x 300 DPI)

For Review Only

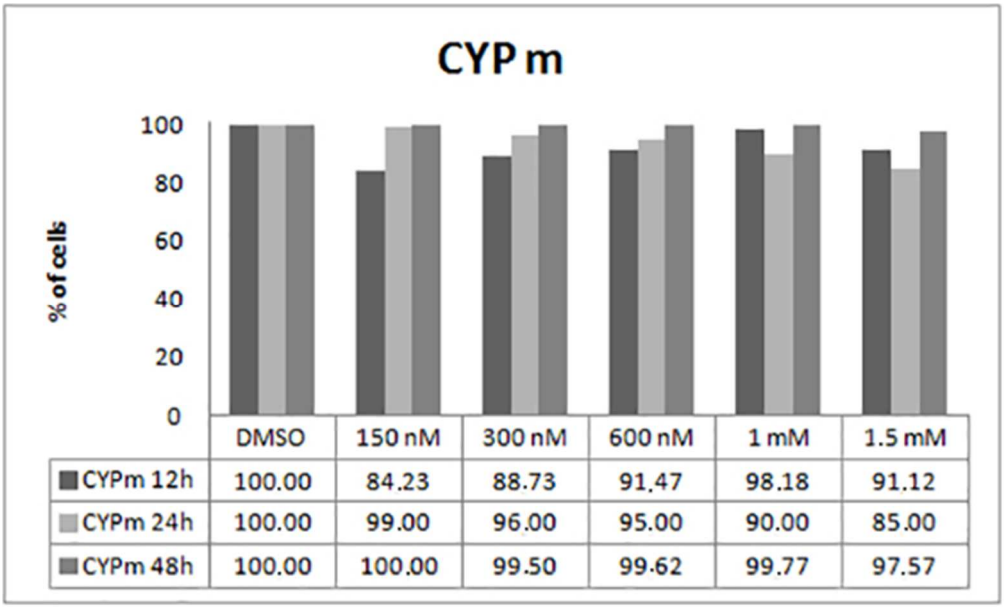
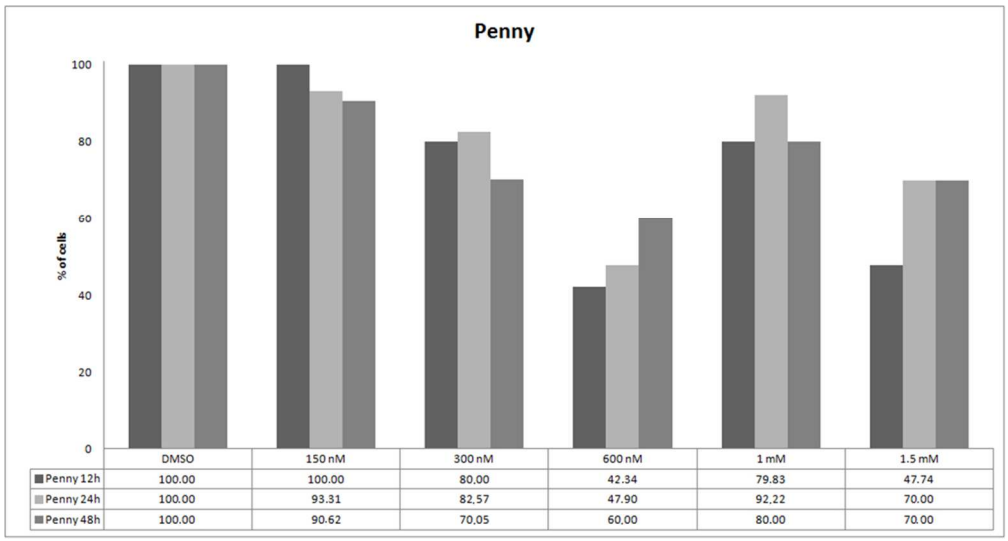


Figure 4B
prolifearting assay
50x30mm (300 x 300 DPI)

For Review Only



	DMSO	150 nM	300 nM	600 nM	1 mM	1.5 mM
Penny 12h	100.00	100.00	80.00	42.34	79.83	47.74
Penny 24h	100.00	93.31	82.57	47.90	92.22	70.00
Penny 48h	100.00	90.62	70.05	60.00	80.00	70.00

Figure 4C
proliferating assay
99x53mm (300 x 300 DPI)

For Review Only

Expression of tyrosine kinase receptors PDGFRα, PDGFRβ, VEGFR-2 and CD117 in canine mammary tumours

Gattino F[a*], Maniscalco L[a], Iussich S[a], Biasato I[a], Martano M[a], Morello E[a], Gola C[a], Millan Y[b], Saeki K[c], Buracco P[a], De Las Mulas J[b] and De Maria R[a]

[a] *Department of Veterinary Sciences, University of Turin, Largo Braccini 2 Grugliasco*

[b] *Department of Comparative Pathology, Veterinary Medicine Faculty, University of Córdoba, Córdoba, Spain*

[c] *Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Japan*

* Corresponding author. Tel +39 011 6709037

E-mail address: francesca.gattino@unito.it

Confidential: For Review Only

Summary

Canine mammary tumours (CMTs) are one of the most common malignancies in bitches. Platelet-derived growth factor receptors (PDGFR)-Alpha and Beta, vascular endothelial growth factor receptor-2 (VEGFR-2) and CD117 are tyrosine kinase receptors (TKRs) involved in several tumours and represent suitable targets for specific therapy with toceranib phosphate. The purpose of this study was to evaluate the expression of these receptors in the pathogenesis and progression of CMTs. PDGFRα, PDGFRβ, VEGFR-2 and CD117 were expressed in 46/83 (55.4%), 33/83 (39.8%), 46/83 (55.4%) and 32/83 (38.5%) of CMTs, respectively. Immunohistochemical results showed a statistically significant loss of PDGFRα and PDGFRβ expression in simple carcinomas compared to complex/mixed carcinomas. Protein expression by western blot revealed specific bands corresponding to PDGFRα and VEGFR-2 in 3/7 and in 1/7 cell lines. Moreover in vitro treatment showed that toceranib phosphate weakly reduced cell proliferation in one canine mammary cell line. These data should be considered for possible therapeutical approaches with specific tyrosine kinase inhibithors (TKI) in this tumor.

Confidential: For Review Only

Introduction

Canine mammary tumours (CMTs) are one of the most common tumours that occur in bitches (Vail and others 2000, Camacho and others 2014). According to the literature, the percentage of the malignant form varies from 40% to 70%, and simple carcinoma represents the most common histotype, followed by mixed and complex tumours (Dobson and others 2002, Salas and others 2015, Vascellari and others 2016). The epithelial component of CMTs has been recognised as a potential model for human breast cancer due to their similar histological and clinical features (Ranieri and others 2013, Liu and others 2014).

Platelet-derived growth factor receptors (PDGFR)-Alpha and Beta, vascular endothelial growth factor receptor-2 (VEGFR-2) and CD117 are tyrosine kinase receptors (TKRs) that are able to activate many of the major cellular signal transduction pathways (Lemmon MA and others 2010) and are involved in both physiological processes and tumoural diseases (Alvarez and others 2006, Demoulin and others 2014).

Numerous studies have demonstrated the over-expression or activation of PDGFRs, VEGFR-2 and CD117 in several canine tumours (Maniscalco and others 2013, Abou Asa and others 2015, Iussich and others 2016). To the author's knowledge, there are still no data regarding the expression of PDGFRs in CMTs, although VEGFR-2 is expressed in 58% of CMTs and is statistically associated with VEGF immunoreactivity in cancer cells (Millanta and others 2006, Santos and others 2014). Further, CD117 is reported to be expressed in 70% of malignant CMTs, which suggests that it plays a role in inflammatory and high-grade canine mammary carcinomas (Brunetti and others 2014). In human breast cancer, PDGFRs and CD117 are involved in cellular differentiation and invasion and in the tumoural microenvironment (Pinto and others 2014); thus, the potential use of targeted therapy has been suggested for this tumour (Zhu and others 2014). The use of TKR inhibitors (TKIs) in human and veterinary medicine depends on the receptor expression, activation and/or mutation, which are usually evaluated in tissues before treatment (Downing and others 2002, Amagai and others 2013). The TKRs evaluated in this study represent an attractive target for

Confidential: For Review Only

toceranib phosphate (Zoetis), which is a veterinary TKI that selectively inhibits VEGFR-2, PDGFRs and CD117 and is currently used for the treatment of mast cell tumours and various other neoplasms, including anal sac carcinomas, head and neck, thyroid carcinomas (London and others 2003 and 2012, Gardner and others 2015). The aims of the present study are to characterise the expression of PDGFRα, PDGFRβ, VEGFR-2 and CD117 in spontaneous CMTs, to evaluate the in vitro effect of toceranib phosphate on canine carcinoma mammary cell lines and to provide information regarding TKRs as molecular targets in this tumour.

Materials and Methods

Sample collection

Eighty-three spontaneous malignant CMTs, 16 benign CMTs, seven hyperplastic mammary glands, and four normal mammary glands were retrieved from a retrospective study at the Hospital of the Department of Veterinary Sciences of the University of Turin; the normal/hyperplastic glands were obtained from healthy dogs during necropsy. Retrospective collection and analysis on CMTs samples was approved by the Ethical Review Committee of the Department of Veterinary Science.

Histological diagnosis

Samples were fixed in 10.0% buffered neutral formalin solution for at least 24 h, embedded in paraffin wax blocks, cut into 4-µm-thickslices and stained with haematoxylin and eosin (HE). CMTs were histologically classified according to Goldschmidt and others (2011).

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was carried out on two sections of 4-µm formalin-fixed paraffin blocks. Primary antibodies (Table 1) were detected using the avidin-biotin peroxidase complex technique with the Vectastain Elite ABC Kit (Vector Laboratories). Canine skin, prostatic carcinoma, normal spleen and mast cell tumour were used as external positive controls for

Confidential: For Review Only

PDGFRα, PDGFRβ, VEGFR-2 and CD117, respectively. For the negative controls, the previous external positive controls were incubated with normal rabbit IgG (sc-2027, St. Crutz Biotechnology). Immunolabelled slides were randomised and masked for blind examination, which was independently performed by three observers (L.M., S.I., Y.M). When there was a disagreement (<5% of the slides), a consensus among the three observers was reached using a multihead microscope. Cytoplasmic immunolabelling of PDGFRs and VEGFR-2 was evaluated in neoplastic and stromal cells (fibroconnective tissue within and surrounding the tumour) separately using the scoring system adopted by Donnem and others 2008 and 2010.

CD117 immunoreactivity was evaluated applying the method adopted by Brunetti and others (2014) as follows: score 0 (absence of positive cells), score 1 (1-19.0% of labelled cells), score 2 (20-49.0% of labelled cells), and score 3 (>50.0% of labelled cells); samples with scores of 1, 2 or 3 were considered positive.

Canine mammary carcinoma (CMC) cell lines

Two canine primary (CYPp, CTB) and one metastatic (CYPm) mammary carcinoma cell lines were provided by the University of Veterinary Medicine of Tokyo, and CF33 cell line was obtained by the American Type Culture Collection (ATCC). All cell lines were cultured as previously reported (Murai and others 2012).

Establishment of Chicca, Lilly, Milly and NEM cell lines

Tissue samples from three dogs with spontaneous grade III simple carcinomas that were surgically treated (Chicca, Lilly, Milly) and from normal mammary tissue (NEM) were collected. After manual disaggregation, tissue fragments from Chicca, Lilly and Milly were digested at 37 °C for 30-60 min in sterile phosphate buffered saline (PBS) containing 0.25 mg/mL collagenase type IA (Sigma-Aldrich) and were then centrifuged and suspended in Dulbecco's modified Eagle medium. For the "Lilly" cell line, 10 µg/mL insulin (Sigma-Aldrich) was added. The NEM cell line was

Confidential: For Review Only

established according to the method presented in the literature (Sánchez-Céspedes and others 2013), and monodispersed cells were grown in Dulbecco's modified Eagle's Medium/Nutrient Mixture F12 Ham (DMEM/F12; Sigma–Aldrich) supplemented with 5% foetal calf serum (FCS), 5000 IU/mL penicillin, 5 mg/mL streptomycin, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone and 10 ng/mL choler toxin. To confirm the epithelial cells of origin, monoclonal mouse anti-cytokeratin (CK) 5 antibody (clone PCK103; isotype IgG_1; Euro-Diagnostica; diluted 1:10) and polyclonal rabbit anti-CK14 antibody (Covance Research; diluted 1:500) were used (data available from the authors).

Western blot analysis

Western blot (WB) analysis was carried out on the protein lysate obtained from previous normal and CMCs (canine mammary carcinoma) cell lines as previously described (Maniscalco and others 2015). After transferring into Hybond-C Extra membranes (American Biosciences), they were incubated with primary antibodies (Table 1). The membranes were then incubated with a secondary horseradish peroxidase (HRP)-linked antibody and subsequently with enhanced chemiluminescence reagent (Super Signal West Pico Mouse IgG Detection Kit, Thermo Scientific).

Immunocytochemistry

CMCc cell lines were grown into wells of eight well chamber slides (Nalgene) at a confluence of 50%. After adhesion and medium removing, the cells were fixed in methanol for 10 minutes at room temperature and washed three times in PBS (phosphate buffer solution) for a total of 15 minutes. After washing, the cells were incubated with primary antibodies (Table 1) according to the previous procedure.

Proliferation assay after in vitro inhibition with toceranib phosphate

Confidential: For Review Only

The CYPm and CTB cell lines overexpressing the highest level of PDGFRA and VEGFR-2 were selected for an in vitro test. A canine osteosarcoma cell line (Penny), expressing high levels of PDGFRs, was used as positive control (Maniscalco and others 2013). First, 10000 cells from each cell line were seeded in 96-well plates, allowed to attach overnight and treated with different concentrations of toceranib (150 nM, 300 nM, 600 nM, 1 μM, 1.5 μM) diluted in Dimethyl sulfoxide for 12, 24 and 48 h. To measure cell proliferation and cytotoxicity, the Cell Counting Kit-8 (Enzo Life Sciences), a colorimetric semi-quantitative assay kit, was used. The experiment was performed in triplicate and repeated three times.

Statistical analysis

IHC results were grouped into contingency tables and analysed using Fisher's exact test or χ^2 test. Proliferating data were analysed using Student's t test. Data were analysed using MedCalc Statistical Software version 13.3 (MedCalc Software bvba). $P < 0.05$ was considered statistically significant.

Results

According to the histological classification of Goldschmidt and others (2011), 50.6% of the cases were classified as simple carcinoma, 30.1% as complex carcinoma and 19.3% as mixed carcinoma. Among them, 28.9% (24/83) were grade I, 41.0% (34/83) were grade II and 30.1% (25/83) were grade III. Immunohistochemical results revealed that none of the four normal mammary samples expressed PDGFRs and only one hyperplastic mammary tissue sample expressed PDGFRs in both stromal and epithelial cells; VEGFR-2 and CD117 positivity was present in 63.6% (7/11) and 18.18% (2/11) of normal and hyperplastic samples both in epithelial and stromal cells, respectively. Benign tumours expressed PDGFRα, PDGFRβ and VEGFR-2 in 54.5%, 54.5% and 62.5% of the epithelial compartment, respectively, and in 18.0%, 18.0% and 37.5% of the stromal compartment, respectively. CD117 immunolabelling was negative in all benign tumours. Immunohistochemical

Confidential: For Review Only

results regarding malignant CMTs are summarised in Tables 2 and 3, and immunohistochemistry pictures are shown in Fig. 1. As shown in Table 2, we found that in malignant tumours, epithelial cells showed significantly lower immunolabelling for PDGFRα and PDGFRβ (P <0.05) in simple carcinomas (16/83 and 12/83) compared to complex and mixed tumours (30/83 and 21/83) respectively.

Moreover, in the stromal compartment, PDGFRβ showed significantly lower immunolabelling in simple carcinomas compared to complex and mixed carcinomas (P <0.05) (Table 3). In malignant tumours, VEGFR-2 was expressed in 46/83 (55.4%) samples at the epithelial level, whereas in stromal cells it was expressed in 18/83 (21.7%) samples. No statistically significant correlation was found between VEGFR-2 and histological grade. CD117 was negative in 61.4% of the cases. The positive samples were distributed as follows: 28.9% (score 1), 6.1%, (score 2) and 3.6% (score 3); no statistically significant correlation was found among CD117 and histological grade. Protein expression analysis revealed that the CF33, CYPp and CYPm cell lines expressed PDGFRα and that the CTB cell line expressed VEGFR-2, whereas none of the established cell lines expressed PDGFRβ or CD117 (Fig. 2). These data were confirmed by immunocytochemistry performed on CMCc cell lines (Fig. 3). In the in vitro test toceranib phosphate was able to slightly inhibit cell proliferation in the CTB cell line at 600 nM (P =0.04) after 24 h and at 1.5 mM after 24 and 48 h of treatment (Fig. 4A). In the CYPm cell line, inhibition was found only at 1.5 mM after 48 h (Fig. 4B). As shown in Fig. 4C, the Penny cell line, which was used as a control, responded to toceranib phosphate in a range of 150-600 nM corresponding to the IC50 at 12, 24 and 48 h (Liao et al., 2002).

Discussion

CMTs are one of the most common neoplasias in dogs. Many recent efforts have been made to increase the knowledge of the pathogenesis of CMTs and to identify new histological biomarkers for prognosis and specific therapy. Toceranib phosphate is currently used to treat canine mast cell

Confidential: For Review Only

tumours (London and others 2009, Amagai and others 2013, Patruno and others 2014, Gil da Costa and others 2015), but only one study evaluated the efficacy of this drug in CMTs (London and others 2003), where four of the five examined CMTs obtained a biological response to therapy (two partial response and two stable disease); however, no data about TKRs expression in these patients are available.

The present study evaluated the expression of PDGFRα, PDGFRβ, VEGFR-2 and CD117 in a large cohort of CMTs to compare their expression to histological features and to identify suitable biomarkers for specific therapy. Considering the importance of these TKRs in the microenvironment of human breast cancer (Nakopoulou and others 2002, Gujam and others 2014, Paulsson and others 2014, Pinto and others 2014, Dekker and others) their expression in epithelial and stromal cells was evaluated separately. The results of this study demonstrated the tendency of neoplastic cells to lose the expression of PDGFRα and PDGFRβ in simple carcinomas compared to mixed/complex carcinomas. None of the normal mammary glands expressed PDGFRs, but 54.5% of benign tumours were positive. On the basis of these results, it could be assumed that these receptors play different roles in benign and malignant tumours; thus, their possible role in the progression from the benign to malignant phenotype in CMTs needs to be investigated. In particular, considering simple carcinomas, PDGFRα and β are not expressed at the epithelial level in 26/42 and 30/42 (61.9% and 71.4%) of the cases, respectively. These results partially correspond to human breast cancer, where PDGFR β is expressed exclusively in stromal cells in 35% of cases, it represents a negative prognostic factor and is usually correlated with the triple negative phenotype (Jechlinger and others 2006, Frings and others 2013, Plantamura and others 2014), whereas PDGFRα is expressed in 39.2% of breast cancer cases in both epithelial and stromal cells (Carvalho and others 2005). On the basis of the varying results obtained, it was hypothesized that different hormonal responses and regulation in the two species should influence the expression of PDGFRs in canine simple carcinoma but further investigations are needed (Peña and others 2014).

Confidential: For Review Only

Similar to what is described in the veterinary literature, VEGFR-2 expression was slightly increased in malignant forms, but no statistical correlation was found with histological grade ($P>0.05$). Our data are in contrast to those of Restucci and others (2004) and Diessler and others (2016), who reported a correlation of VEGFR-2 with malignant forms and microvascular density. Nevertheless, our data are similar to those obtained in humans, where the immunoistochemical expression of VEGFR-2 ranged between 63.0% and 69.0% (Rydén and others 2005) and was generally related to an aggressive phenotype and a poor prognosis (Johansson and others 2012). Finally, CD117 expression did not reveal a statistical association with tumour histotype; these data are not in accordance with those of Brunetti and others (2014), who demonstrated a statistical association between CD117 expression in malignant tumours with respect to benign tumours. However, our data are in agreement with the human literature, where high-grade carcinomas do not express CD117 (Tomasino and others 2009, Kondi-Pafiti and others 2010). Western blot analysis confirmed the antibody specificity and showed that these receptors are poorly expressed in carcinoma cell lines analyzed. Finally, the in vitro assay suggested that the response to drugs is strictly dependent on the presence of specific TKRs on the cellular membrane surface. The results of the present study show that PDGFRα and PDGFRβ tend to lose their expression in simple carcinomas and on the basis of preliminary results we can assume that the biological effect of toceranib phosphate on CMCs strictly depends on the cellular expression of specific TKRs. These data should be considered before a specific medical treatment is initiated, although further clinical studies in this direction are needed.

Acknowledgements

The authors would like to thank Dr. Edge and all of the personnel at Zoetis for supporting the present study by providing toceranib phosphate (CPT number WI128659). We also greatly appreciate the help of Dr. Sara Verganti and Neal Bryant for their assistance with the English language and comments on this manuscript.

Confidential: For Review Only

References

ABOU ASA, S., MORI, T., MARUO, K., KHATER, A., EL-SAWAK, A., ABD EL-AZIZ, E., YANAI, T., SAKAI, H. (2015) Analysis of genomic mutation and immunohistochemistry of platelet-derived growth factor receptors in canine vascular tumours. *Veterinary and Comparative Oncology* **13**, 237-245.

ALVAREZ, R.H., KANTARJIAN, H.M., CORTES, J.E. (2006) Biology of platelet-derived growth factor and its involvement in disease. *Mayo Clinic Proceeding* **81**, 1241-1257.

AMAGAI, Y., TANAKA, A., MATSUDA, A., JUNG, K., OIDA, K., NISHIKAWA, S., JANG, H., MATSUDA, H. (2013) Heterogeneity of internal tandem duplications in the c-kit of dogs with multiple mast cell tumours. *Journal of Small Animal Practice* **54**, 377-380.

BRUNETTI, B., BEHA, G., BENAZZI, C., BONDIN, V., DE TOLLA, L., SARLI, G. (2014) CD117 expression influences proliferation but not survival in canine mammary tumours. *Journal of Comparative Pathology* **151**, 202-206.

CAMACHO, L., PEÑA, L., GIL, A.G., MARTÍN-RUIZ, A., DUNNER, S., ILLERA, J.C. (2014) Immunohistochemical vascular factor expression in canine inflammatory mammary carcinoma. *Veterinary Pathology* **51**, 737-748.

CARVALHO, I., MILANEZI, F., MARTINS, A., REIS, R.M., SCHMITT, F. (2005) Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression. *Cancer Research* **7**, 788-795.

DEKKER, T.J., CHAREHBILI, A., SMIT, V.T., TEN DIJKE, P., KRANENBARG, E.M., VAN DE VELDE, C.J., NORTIER, J.W., TOLLENAAR, R.A., MESKER, W.E., KROEP, J.R. (2015) Disorganised stroma determined on pre-treatment breast cancer biopsies is associated with poor response to neoadjuvant chemotherapy: Results from the NEOZOTAC trial. *Molecular Oncology* **9**, 1120-1128.

DE LAS MULAS J., MILLAN Y., DIOS R. (2005) A prospective analysis of immunohistochemically determined estrogen receptor and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog. *Veterinary Pathology* **42**, 200–212.

DEMOULIN, J.B., ESSAGHIR, A. (2014) PDGF receptor signaling networks in normal and cancer cells. *Cytokine and Growth Factor Reviews* **25**, 273-283.

DIESSLER, M.E., CASTELLANO, M.C., PORTIANSKY, E.L., BURNS, S., IDIART, J.R. (2016) Canine mammary carcinomas: influence of histological grade, vascular invasion, proliferation, microvessel density and VEGFR2 expression on lymph node status and survival time. *Veterinary and Comparative Oncology* (doi: 10.1111/vco.12189) .

DONNEM, T., AL-SAAD, S., AL-SHIBLI, K., ANDERSEN, S., BUSUND, L.T., BREMNES, R.M. (2008) Prognostic impact of platelet-derived growth factors in non-small cell lung cancer tumour and stromal cells. *Journal of Thoracic Oncology* **3**, 963-970.

DONNEM, T., AL-SHIBLI, K., ANDERSEN, S., AL-SAAD, S., BUSUND, LT., BREMNES, RM. (2010) Combination of low vascular endothelial growth factor A (VEGF-A)/VEGF receptor

For Peer Review Only Confidential

2 expression and high lymphocyte infiltration is a strong and independent favorable prognostic factor in patients with non small cell lung cancer. *Cancer* **15**, 4318-4325.

DOBSON J.M., SAMUEL S., MILSTEIN H., ROGERS K., WOOD J.L. (2002) Canine neoplasia in the UK: estimates of incidence rates from a population of insured dogs. *Journal of Small Animal Practice*, **43**, 240–246.

DOWNING, S., CHIEN, M.B., KASS, P.H., MOORE, P.E., LONDON, C.A. (2002) Prevalence and importance of internal tandem duplications in exons 11 and 12 of c-kit in mast cell tumours of dogs. *American Journal of Veterinary Research* **63**, 1718-1723.

FRINGS, O., AUGSTEN, M., TOBIN, N.P., CARLSON, J., PAULSSON, J., PENA, C., OLSSON, E., VEERLA, S., BERGH, J., OSTMAN, A., ET AL. (2013) Prognostic significance in breast cancer of a gene signature capturing stromal PDGF signaling. *The American Journal of Pathology,* **182**, 2037-2047.

GARDNER, H.L., LONDON, C.A., PORTELA, R.A., NGUYEN, S., ROSENBERG, M.P., KLEIN, M.K., CLIFFORD, C., THAMM, D.H., VAIL, D.M., BERGMAN, P., CRAWFORD-JAKUBIAK, M., HENRY, C., LOCKE J., GARRET, LD. (2015) Maintenance therapy with toceranib following doxorubicin-based chemotherapy for canine splenic hemangiosarcoma. *BMC Veterinary Research* (doi: 10.1186/s12917-015-0446-1) .

GIL DA COSTA, R.M. (2015) C-kit as a prognostic and therapeutic marker in canine cutaneous mast cell tumours: From laboratory to clinic. *The Veterinary Journal* **205**, 5-10.

GOLDSCHMIDT, M., PEÑA, L., RASOTTO, R., ZAPPULLI, V. (2011) Classification and grading of canine mammary tumours. *Veterinary Pathology* **48**, 117-131.

GUJAM, F.J., EDWARDS, J., MOHAMMED, Z.M., GOING, J., MCMILLAN, D.C. (2014) The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with operable ductal breast cancer. *British Journal of Cancer* **111**, 157-165.

IUSSICH, S., MANISCALCO, L., DI SCIUVA, A., IOTTI, B., MORELLO, E., MARTANO, M., GATTINO, F., BURACCO, P., DE MARIA, R. (2016) PDGFRs expression in dogs affected by malignant oral melanomas: correlation with prognosis. *Veterinary and Comparative Oncology* (doi: 10.1111/vco.12190).

JECHLINGER, M., SOMMER, A., MORIGGL, R., SEITHER, P., KRAUT, N., CAPODIECCI, P., DONOVAN, M., CORDON-CARDO, C., BEUG, H., GRÜNERT, S. (2006) Autocrine PDGFR signaling promotes mammary cancer metastasis. *Journal of Clinical Investigation* **116,** 1561-1570.

JOHANSSON, I., AALTONEN, K.E., EBBESSON, A., GRABAU, D., WIGERUP, C., HEDENFALK, I., RYDÉN, L.(2012) Increased gene copy number of KIT and VEGFR2 at 4q12 in primary breast cancer is related to an aggressive phenotype and impaired prognosis. *Genes Chromosomes Cancer* **51**, 375-383.

KONDI-PAFITI, A., ARKADOPOULOS, N., GENNATAS, C., MICHALAKI, V., FRANGOU-PLEGMENOU, M., CHATZIPANTELIS, P. (2010) Expression of c-kit in common benign and malignant breast lesions. *Tumori* **96**, 978-984.

Confidential - For Review Only

LEMMON M.A., SCHLESSINGER J. (2010) Cell Signalling by receptor tyrosine kinases. *Cell* **25**, 1117-34.

LIAO, A.T., CHIEN, M.B., SHENOY, N., MENDEL, D.B., MCMAHON, G., CHERRINGTON, J.M., LONDON, C.A. (2002) Inhibition of constitutively active forms of mutant kit by multitargeted indolinone tyrosine kinase inhibitors. *Blood* **100**, 585-593.

LIU, D., XIONG, H., ELLIS, A.E., NORTHRUP, N.C., RODRIGUEZ, C.O., O'REGAN, R.M., DALTON, S., ZHAO, S. (2014) Molecular homology and difference between spontaneous canine mammary cancer and human breast cancer. *Cancer Research* **74**, 5045-5056.

LONDON, C.A., HANNAH, A.L., ZADOVOSKAYA, R., CHIEN, M.B., KOLLIAS-BAKER, C., ROSENBERG, M., DOWNING, S., POST, G., BOUCHER, J., SHENOY, N. MENDEL, DB., MCMACHON, G., CHERRINGTON JM. (2003) Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. *Clinical Cancer Research* **9**, 2755-2768.

LONDON, C.A., MALPAS, P.B., WOOD-FOLLIS, S.L., BOUCHER, J.F., RUSK, A.W., ROSENBERG, M.P., HENRY, C.J., MITCHENER, K.L., KLEIN, M.K., HINTERMEISTER, J.G., BERGMAN, PJ., COUTO, GC., MAULDIN, GN., MICHELS, GM. (2009) Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumour following surgical excision. *Clinical Cancer Research* **15**, 3856-3865.

LONDON, C., MATHIE, T., STINGLE, N., CLIFFORD, C., HANEY, S., KLEIN, M.K., BEAVER, L., VICKERY, K., VAIL, D.M., HERSHEY, B., ET AL., (2012) Preliminary evidence for biologic activity of toceranib phosphate (Palladia(®)) in solid tumours. *Veterinary and Comparative Oncology* **10**, 194-205.

MANISCALCO, L., IUSSICH, S., MORELLO, E., MARTANO, M., BIOLATTI, B., RIONDATO, F., DELLA SALDA, L., ROMANUCCI, M., MALATESTA, D., BONGIOVANNI, L., TIRRITO, F., GATTINO, F., BURACCO, P., DE MARIA, R. (2013) PDGFs and PDGFRs in canine osteosarcoma: new targets for innovative therapeutic strategies in comparative oncology. *The Veterinary Journal* **195**, 41-47.

MANISCALCO, L., IUSSICH, S., MORELLO, E., MARTANO, M., GATTINO, F., MIRETTI, S., BIOLATTI, B., ACCORNERO, P., MARTIGNANI, E., SÁNCHEZ-CÉSPEDES, R., BURACCO, P., DE MARIA, R. (2015) Increased expression of insulin-like growth factor-1 receptor is correlated with worse survival in canine appendicular osteosarcoma. *The Veterinary Journal* **205**, 272-280.

MILLANTA, F., SILVESTRI, G., VASELLI, C., CITI, S., PISANI, G., LORENZI, D., POLI, A. (2006) The role of vascular endothelial growth factor and its receptor Flk-1/KDR in promoting tumour angiogenesis in feline and canine mammary carcinomas: a preliminary study of autocrine and paracrine loops. *Research in Veterinary Science* **81**, 350-357.

MURAI, K., NAKAGAWA, T., ENDO, Y., KAMIDA, A., YOSHIDA, K., MOCHIZUKI, M., NISHIMURA, R., SASAKI, N. (2012) Establishment of a pair of novel cloned tumour cell

Confidential: For Review Only

lines with or without metastatic potential from canine mammary adenocarcinoma. *Research in Veterinary Science* **93**, 468-472.

NAKOPOULOU, L., STEFANAKI, K., PANAYOTOPOULOU, E., GIANNOPOULOU, I., ATHANASSIADOU, P., GAKIOPOULOU-GIVALOU, H., LOUVROU, A. (2002). Expression of the vascular endothelial growth factor receptor-2/Flk-1 in breast carcinomas: correlation with proliferation. Human Pathology 33, 863-870.

PATRUNO, R., MARECH, I., ZIZZO, N., AMMENDOLA, M., NARDULLI, P., GADALETA, C., INTRONA, M., CAPRIUOLO, G., RUBINI, R.A., RIBATTI, D., ET AL. (2014) c-Kit expression, angiogenesis, and grading in canine mast cell tumour: a unique model to study c-Kit driven human malignancies. *BioMed Research International* (doi: 10.1155/2014/730246).

PAULSSON, J., MICKE, P. (2014) Prognostic relevance of cancer-associated fibroblasts in human cancer. *Seminars in Cancer Biology* **25**, 61-68.

PEÑA, L., GAMA, A., GOLDSCHMIDT, M.H., ABADIE, J., BENAZZI, C., CASTAGNARO, M., DÍEZ, L., GÄRTNER, F., HELLMÉN, E., KIUPEL, M., ET AL. (2014) Canine mammary tumours: a review and consensus of standard guidelines on epithelial and myoepithelial phenotype markers, HER2, and hormone receptor assessment using immunohistochemistry. *Veterinary Pathology* **51**, 127-145.

PINTO, M.P., DYE, W.W., JACOBSEN, B.M., HORWITZ, K.B. (2014) Malignant stroma increases luminal breast cancer cell proliferation and angiogenesis through platelet-derived growth factor signaling. *BMC Cancer* (doi: 10.1186/1471-2407-14-735).

PLANTAMURA, I., CASALINI, P., DUGNANI, E., SASSO, M., D'IPPOLITO, E., TORTORETO, M., CACCIATORE, M., GUARNOTTA, C., GHIRELLI, C., BARAJON, I., ET AL. (2014) PDGFRβ and FGFR2 mediate endothelial cell differentiation capability of triple negative breast carcinoma cells. *Molecular Oncology* 8, 968-981.

RANIERI, G., PANTALEO, M., PICCINNO, M., RONCETTI, M., MUTINATI, M., MARECH, I., PATRUNO, R., RIZZO, A., SCIORSCI, R.L. (2013) Tyrosine kinase inhibitors (TKIs) in human and pet tumours with special reference to breast cancer: a comparative review. *Critical Reviews in Oncology/Hematology* **88**, 293-308.

RESTUCCI, B., BORZACCHIELLO, G., MAIOLINO, P., MARTANO, M., PACIELLO, O., PAPPARELLA, S. (2004) Expression of vascular endothelial growth factor receptor Flk-1 in canine mammary tumours. *Journal of Comparative Pathology* **130**, 99-104.

RYDÉN, L., STENDAHL, M., JONSSON, H., EMDIN, S., BENGTSSON, N.O., LANDBERG, G. (2005) Tumour-specific VEGF-A and VEGFR2 in postmenopausal breast cancer patients with long-term follow-up. Implication of a link between VEGF pathway and tamoxifen response. *Breast Cancer Research Treatment* **89**, 135-143.

SÁNCHEZ-CÉSPEDES, R., MANISCALCO, L., IUSSICH, S., MARTIGNANI, E., GUIL-LUNA, S., DE MARIA, R., DE LAS MULAS, J., MILLÁN, Y. (2013) Isolation, purification, culture and characterisation of myoepithelial cells from normal and neoplastic canine mammary glands using a magnetic-activated cell sorting separation system. *The Veterinary Journal* **197**, 474-482.

Confidential: For Review Only

SALAS, Y., MÁRQUEZ, A., DIAZ, D., ROMERO, L. (2015) Epidemiological Study of Mammary Tumours in Female Dogs Diagnosed during the Period 2002-2012: A Growing Animal Health Problem. *PLoS One* (doi: e0127381).

SANTOS, A., LOPES, C., GÄRTNER, F., MATOS, A.J. (2014) VEGFR-2 expression in malignant tumours of the canine mammary gland: a prospective survival study. *Veterinary and Comparative Oncology* (doi: 10.1111/vco.12107).

TOMASINO, R.M., MORELLO, V., GULLO, A., POMPEI, G., AGNESE, V., RUSSO, A., RINALDI, G. (2009) Assessment of "grading" with Ki-67 and c-kit immunohistochemical expressions may be a helpful tool in management of patients with flat epithelial atypia (FEA) and columnar cell lesions (CCLs) on core breast biopsy. *Journal of Cellular Physiology* **221**, 343-349.

VAIL, D.M., MACEWEN, E.G. (2000) Spontaneously occurring tumours of companion animals as models for human cancer. *Cancer Investigation* **18**, 781-792.

VASCELLARI, M., CAPELLO, K., CARMINATO, A., ZANARDELLO, C., BAIONI, E., MUTINELLI, F. (2016) Incidence of mammary tumours in the canine population living in the Veneto region (Northeastern Italy): Risk factors and similarities to human breast cancer. *Preventive Veterinary Medicine* **126**, 183-189.

ZHU, Y., WANG, Y., GUAN, B., RAO, Q., WANG, J., MA, H., ZHANG, Z., ZHOU, X. (2014) C-kit and PDGFRA gene mutations in triple negative breast cancer. *International Journal of Clinical Experimental Pathology* **7**, 4280-4285.

Confidential: For Review Only

Table 1

Sources, specificity and dilutions of the antibodies employed in immunohistochemistry (IHC) and Western Blot (WB).

Antibody	Type	Source	Specificity[a]	IHC	WB
VEGFR-2	Rabbit polyclonal	Santa Crutz Biotechnology	m	1:150	1:1000
PDGFRα	Rabbit polyclonal	Santa Crutz Biotechnology	h	1:100	1:1000
PDGFRβ	Rabbit polyclonal	Santa Crutz Biotechnology	h, m, r	1:200	/
PDGFRβ	Rabbit polyclonal	Cell Signaling Technology	h, m, r	/	1:1000
CD117	Rabbit polyclonal	DAKO	h	1:400	1:1000

[a]Human (h), mouse (m) and rat (r).

Confidential: For Review Only

Table 2

Relationships between tumour characteristics and Tirosine Kinase Receptors expressions in neoplastic cells

Clinicopathologic characteristics	PDGFRα positive *n* (%)	PDGFRα negative *n* (%)	*P value*	PDGFRβ positive *n* (%)	PDGFRβ negative *n* (%)	*P value*	VEGFR-2 positive *n* (%)	VEGFR-2 negative *n* (%)	*P value*	CD117 positive *n* (%)	CD117 negative *n* (%)	*P value*
Simple carcinomas	16/83 (9.6)	26/83 (24.1)	0.002	12/83 (14.4)	30/83 (36.2)	0.03	22/83 (26.5)	20/83 (24.1)	> 0.05	20/83 (24.1)	22/83 (26.5)	> 0.05
Complex and mixed carcinomas	30/83 (45.8)	11/83 (20.5)		21/83 (25.3)	20/83 (24.1)		24/83 (28.9)	17/83 (20.5)		12/83 (14.4)	29/83 (34.9)	
Total	46	37		33	50		46	37		32	51	
Grade I	11/83 (13.2)	13/83 (15.7)	> 0.05	11/83 (13.2)	13/83 (15.7)	> 0.05	14/83 (16.9)	10/83 (12.0)	> 0.05	10/83 (12.0)	14/83 (16.9)	> 0.05
Grade II	21/83 (25.3)	13/83 (15.7)		16/83 (19.3)	18/83 (21.7)		20/83 (24.1)	14/83 (16.9)		14/83 (16.9)	20/83 (24.1)	
Grade III	14/83 (16.9)	11/83 (13.2)		6/83 (7.2)	19/83 (22.9)		12/83 (14.4)	13/83 (15.7)		8/83 (9.6)	17/83 (20.5)	
Total	46	37		33	50		46	37		32	51	
Dimension <3cm	23/83 (27.8)	18/83 (21.7)	> 0.05	18/83 (21.7)	23/83 (27.8)	> 0.05	23/83 (27.8)	18/83 (21.5)	> 0.05	16/83 (19.3)	25/83 (30.1)	> 0.05
Dimension 3-5cm	14/83 (16.9)	11/83 (13.2)		8/83 (9.6)	17/83 (20.5)		15/83 (18.7)	10/83 (12.0)		10/83 (12.0)	15/83 (18.7)	
Dimension >5cm	9/83 (10.8)	8/83 (9.6)		7/83 (8.4)	10/83 (12.0)		8/83 (9.6)	9/83 (10.8)		6/83 (7.2)	11/83 (13.2)	
Total	46	37		33	50		46	37		32	51	

Confidential: For Review Only

Table 3

Relationships between tumour characteristics and Tirosine Kinase Receptors expressions in stromal cells

	PDGFRα positive *n* (%)	PDGFRα negative *n* (%)	*P value*	PDGFRβ positive *n* (%)	PDGFRβ negative *n* (%)	*P value*	VEGFR-2 positive *n* (%)	VEGFR-2 negative *n* (%)	*P value*
Simple carcinomas	8/83 (9.6)	34/83 (41.0)	> 0.05	5/83 (6.0)	37/83 (44.6)	0.01	9/83 (10.8)	33/83 (39.9)	> 0.05
Complex and mixed carcinomas	10/83 (12.0)	31/83 (37.4)		14/83 (16.9)	27/83 (32.5)		9/83 (10.8)	32/83 (38.5)	
Total	18	65		19	64		18	65	
Grade I	4/83 (4.8)	20/83 (24.1)	> 0.05	5/83 (6.0)	19/83 (22.9)	> 0.05	8/83 (9.6)	16/83 (19.3)	> 0.05
Grade II	7/83 (8.4)	27/83 (32.6)		11/83 (13.2)	23/83 (27.8)		4/83 (4.8)	30/83 (36.2)	
Grade III	7/83 (8.4)	18/83 (21.7)		3/83 (3.6)	22/83 (26.5)		6/83 (7.2)	19/83 (22.9)	
Total	18	65		19	64		18	65	
Dimension <3cm	7/83 (8.4)	34/83 (41.0)	> 0.05	11/83 (13.2)	30/83 (36.2)	> 0.05	10/83 (12.0)	31/83 (37.4)	> 0.05
Dimension 3-5cm	8/83 (9.6)	17/83 (20.5)		6/83 (7.2)	19/83 (22.9)		3/83 (3.6)	22/83 (26.5)	
Dimension >5cm	3/83 (3.6)	14/83 (16.9)		2/83 (2.2)	15/83 (18.7)		5/83 (6.0)	12/83 (14.4)	
Total	18	65		19	64		18	65	

Confidential: For Review Only

Figure legends

Fig. 1. Simple carcinomas. Picture A and B showed a strong cytoplasmic immunolabelling in the neoplastic cells for platelet-derived growth factor receptors (PDGFR)-α and β, respectively. Pictures C and D showed intermediate immunolabelling in the stromal cells for PDGFRα and β, respectively. Picture E showed a strong cytoplasmic immunolabelling in the neoplastic cells for vascular endothelial growth factor receptor-2 (VEGFR-2) (E) and an intermediate cytoplasmic immunolabelling for CD117 (picture F). Streptavidin-biotin-peroxidase method. Mayer's haematoxylin counterstain. Scale bar 50 µM.

Fig. 2. Western Blot analysis of Platelet-derived growth factor receptors (PDGFR)-α and β and Vascular endothelial growth factor receptor-2 expression in normal epithelial mammary cell lines NEM (lane1), and neoplastic cell lines CYPp (lane 2), CYPm (lane 3), CTB (lane 4), CF33 (lane 5), Lilly (lane 6), Chicca (lane 7), Milly (lane 8) cell lines. Specific molecular weight of protein are indicated as Kilodalton (KDa). Alpha Tubulin expression was used as the loading control.

Fig. 3: Immunocitochemistry against CD117, PDGFRα, PDGFRβ and VEGFR2 on canine mammary carcinoma cell lines. (A) CTB cells stained with CD117 antibody (20X magnification), (B) CypM cells stained with PDGFR β antibody (20X magnification), (C) CypP cells stained with PDGFR α antibody (20X magnification), (D) CTB cells stained with VEGFR2 antibody (20X magnification)

Fig. 4. Evaluation of in vitro response to toceranib phosphate for CTB (Fig. 4A), CYPm (Fig. 4B) and Penny (Fig. 4C) cell lines at different time (12 h, 24 h, 48 h) and concentration (150 nM, 300 nM, 600 nM, 1 µM, 1,5 µM).

Confidential. For Review Only