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Cycle XXVIII

Tirosine Kinase Receptors expression in canine mammary tumours and in vitro and in vivo evaluation of Tirosine Kinases inhibitors biological activity. Evaluation of possible molecular targets in comparative oncology

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CONTENTS

SUMMARY

Canine mammary tumours (CMTs) are one the most frequent neoplasm that occur in entire female dogs. Approximately from 40% to 70% of CMTs are malignant and tend to metastasize; simple carcinoma represents the most common histotype, followed by mixed and complex tumours. Different studies have evaluated the risk to develop a malignant neoplasm in correlation with the hormonal status and it is largely demonstrate that an early neutering is associated with a decrease in incidence. Canine mammary cancer, similar to human, is a heterogeneous group of disease linked to different morphology and biological behavior.

Usually the first therapeutically approach in the veterinary field is surgical but in case of locally aggressive tumours or metastatic disease other treatment are required to control the disease. The radiotherapy and traditional chemotherapy in adjuvant or palliative treatment are extremely discussed and usually not effective in order to control the disease. In the last years, several studies tried to evaluate the hormonal status and receptors expression for therapeutically finalities but with limited success.

Mammary gland tumours can share common features between human and pets and could be excellent models for comparative studies. Indeed, similarities in aberrant tumoural cell division with damaged DNA replication, hypoxia, mutations accumulation and DNA repair genes and in general activated proto-oncogenes were reported in the two species suggesting similar molecular mechanism in the pathogenesis of this tumour in feline and canine species. For this reason and with the aim to provide an adequate support for diagnostic and therapeutic strategies in veterinary medicine, several oncological studies are currently underway in molecular biology.

In particular, changes in genes encoding growth factors and receptors have shown great relevance in cell growth differentiation, proliferation, invasion and metastasis. The Tyrosine Kinase Receptors are an important family of oncogenes playing a relevant role in cancer biology of humans and pets. Among them platelet-derived growth factor receptors (PDGFR)-Alpha and Beta, vascular endothelial growth factor receptor-2 (VEGFR-2) and CD117 are over-expressed and activated in several tumours suggesting the application of specific use of Tyrosine Kinase Inhibitors (TKIs) as masitinib phosphate and toceranib phosphate. The aims of the present study are to characterize the expression of PDGFRα, PDGFRβ, VEGFR-2 and CD117 in canine mammary carcinomas at immunohistochemical and molecular level in order to provide information regarding TKRs as potential prognostic biomarkers and

molecular target in this tumour. Moreover, the biological effect of the toceranib phosphate (Palladia, Zoetis) has been evaluated in in vitro cell lines and in dogs affected by mammary tumors.

CANINE MAMMARY TUMOURS

Canine mammary tumours (CMTs) are one of the most common neoplasm occurring in entire bitches and represent the 50% to 70% of all neoplasm (Vail and MacEwen, 2000; Camacho et al., 2014). Several studies had showed as benign and malignant mammary tumours are not separate entities but part of a biologic and histopathologic process with direct similarities with human breast cancer. Due to these reasons, the mammary gland tumours are a very heterogeneous group of neoplasm showing different hormonal expression, grade of aggressiveness and histotype. According to literature from 40% to 70% of CMTs are malignant and the simple carcinoma represents the most common histotype followed by mixed and complex tumours (Salas et al., 2015; Vascellari et al., 2016).

In particular, the canine simple carcinoma has been recognized as a potential model for human breast cancer, due to some histological and clinical similarities (Ranieri et al., 2013; Liu et al., 2014).

Clinical features and prognosis

Mammary tumours are usually easy to detect through the physical examination, despite this, sometimes the diagnosis is not early. Typically, the majority of CMTs affected the inguinal glands where the tissue is larger and small masses could be detected late in time. Concomitant other neoplastic nodules could be present in a 70% of dogs. The size of the tumour(s) and the clinical presentation (position, adhesion to surrounded tissue) could vary widely. The tumoral size, the lymph node involvement and the stage are the three more important prognostic factors associated with prognosis. The percentage of possible metastasis could be very variable and regional and distant most frequent metastases included lymph nodes, liver, lungs and bone.

Inflammatory carcinoma is a clinical entities characterized by a very inflamed, painful, swollen mass, usually confused with a severe mastitis, with a very high risk of metastasis and a survival time inferior to 60 days (Vail and MacEwen, 2000).

Staging system

Mammary tumours are clinically staged according the T (tumour), N (lymph node), M (metastasis) system. The modified version of the original staging system published by Owens (Owen et al., 1980) currently used, is here reported.

TNM System in mammary gland tumours in dogs

T Tumoral dimension

Tis carcinoma in situ

T0 any evidence of tumour

T1 dimension < 3cm

T2 dimension 3-5 cm

T3 dimension >5cm

T4 inflammatory carcinoma

N Regional lymph nodes

N0 no metastasis

N1 metastasis in regional lymph node

N2 metastasis in regional and not regional lymph nodes

M Distant metastasis

M0 no metastasis

M1 presence of metastasis

In the Fig. 1 and 2 were represented two cases of CMTs of stage T3 N0 M0, in particularly the fig. 2 shown the clinical aspect of an inflammatory carcinoma.

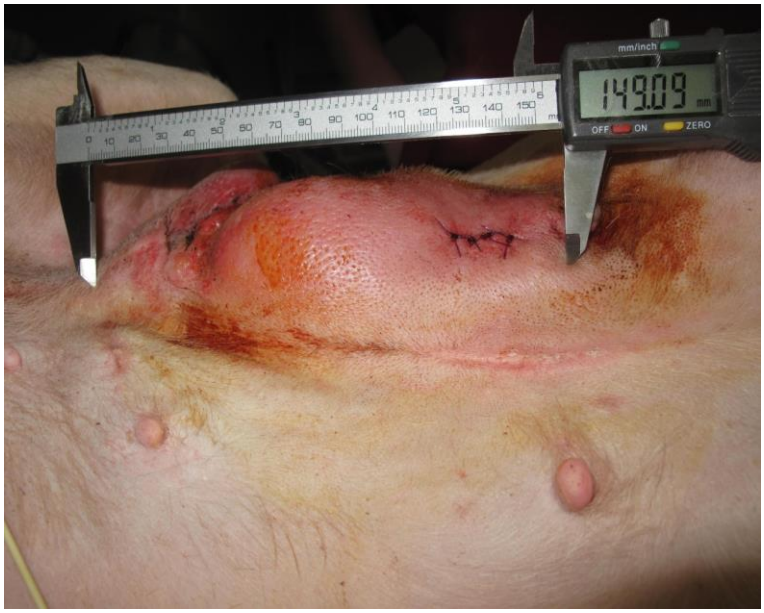


Fig. 1

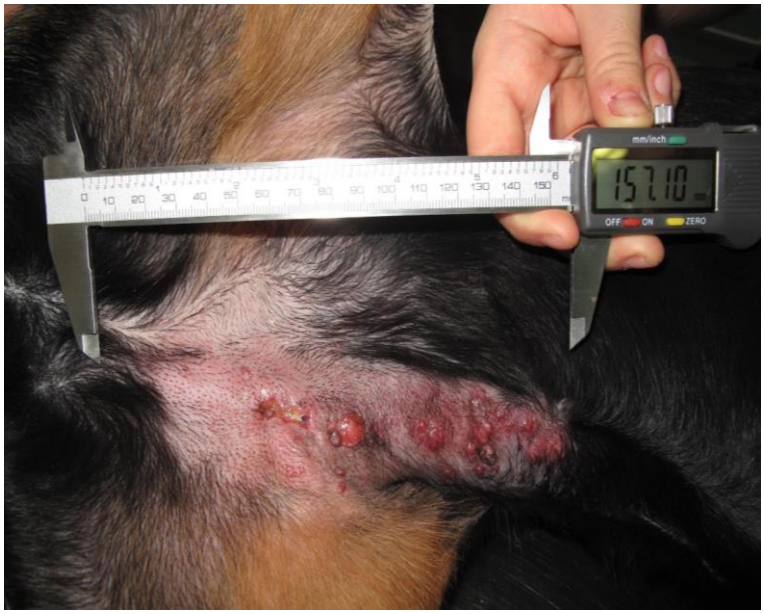


Fig. 2

In the following Fig. 3 and 4 were reported two cases of involvement of the regional lymph node: the evaluation of the dimension is normally calculated in the short axis.

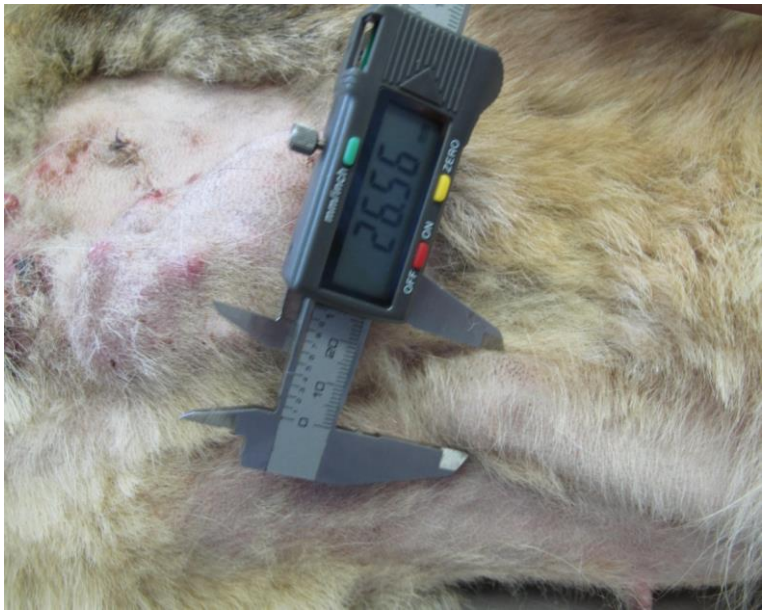


Fig. 3

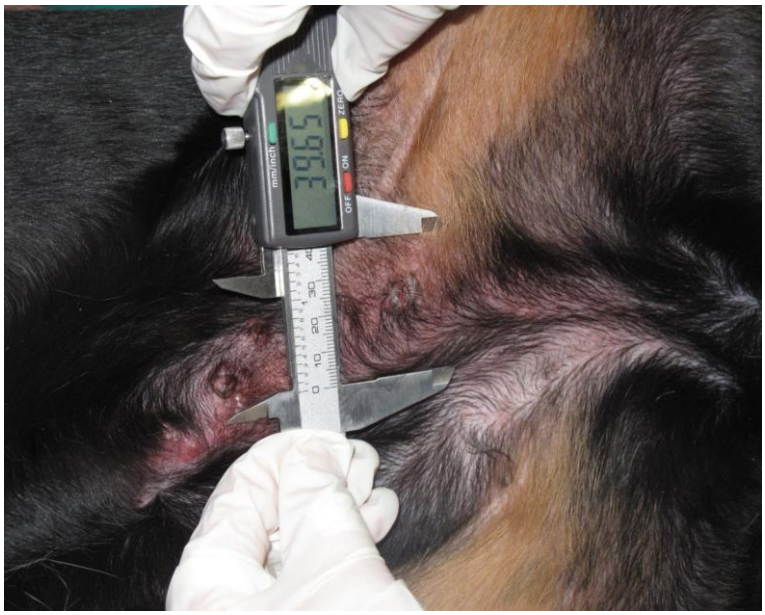


Fig. 4

Finally, the fig. 5 and 6, shown cases of metastasis: in the fig. 5, a sternal metastatic lymph node increased in size is present in the cranial mediastina (cytological confirmed); while in the fig. 6 a diffuse metastatic pattern of the lungs was present.

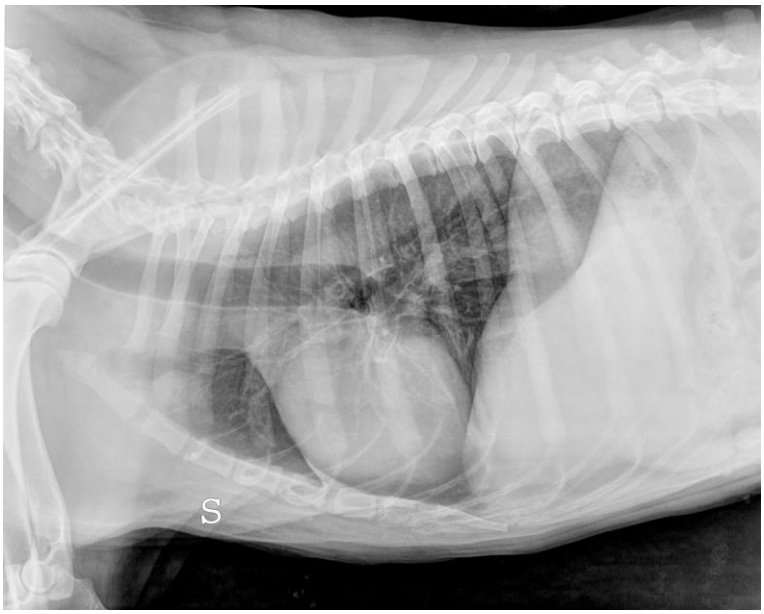


Fig. 5

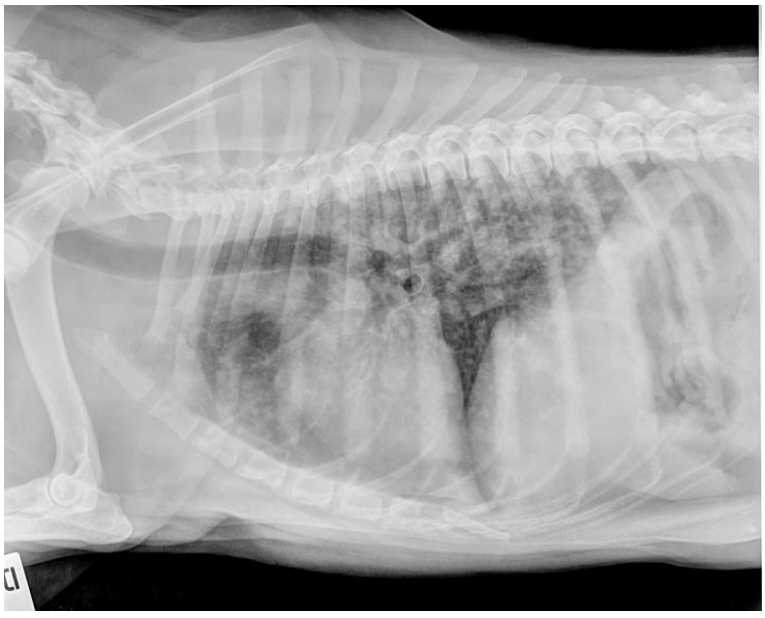


Fig. 6

Histopathology

The first histological classification about histopathology of mammary tumours in dogs and cats rises to 1974 (Hampe et al., 1974) and 1999 (Misdorp et al., 1999). Recently Goldschmidt et al. (Goldschmidt et al., 2011) published the new classification of CMT keeping in consideration both the histological morphology and the prognosis (according the histotype) and trying to explain the different behaviors of these tumours.

The simple carcinomas are neoplasm originating by a single type of cell (epithelial or myoephitelial) while the complex carcinomas can originate by two different kind of cells: the epithelial and the myoepithelial cells. A different entity are the mesenchymal neoplasms that arise from the mesenchymal tissue and are characterized by a rapid growth and early metastatisation.

The histological criteria of malignancy considered are the histological type, the nuclear and cellular pleomorphism, mitotic index, presence of areas of necrosis, peritumoral and lymphatic invasion.

1: Malignant Epithelial Neoplasms

Carcinoma–in situ

Carcinoma–simple

1. Tubular
2. Tubulopapillary
3. Cystic-papillary
4. Cribriform

Carcinoma–micropapillary invasive

Carcinoma–solid

Comedocarcinoma

Carcinoma–anaplastic

Carcinoma arising in a complex adenoma/mixed tumor

—The benign counterpart is still detectable in the section.

Carcinoma–complex type

—The epithelial component is malignant, and the myoepithelium is benign.

Carcinoma and malignant myoepithelioma

—The epithelial and myoepithelial components are malignant.

Carcinoma–mixed type

—The epithelial component is malignant; the myoepithelial mesenchymal component is benign; and the mesenchymal component is cartilage or bone.

Ductal carcinoma–malignant counterpart of ductal adenoma

Intraductal papillary carcinoma–malignant counterpart of intraductal papillary adenoma

2: Malignant Epithelial Neoplasms—Special Types

Squamous cell carcinoma

Adenosquamous carcinoma

Mucinous carcinoma

Lipid-rich (secretory) carcinoma

Spindle cell carcinomas

Malignant myoepithelioma

Squamous cell carcinoma–spindle cell variant

Carcinoma–spindle cell variant

Inflammatory carcinoma (see Inflammatory Carcinoma section)

3: Malignant Mesenchymal Neoplasms—Sarcomas

Osteosarcoma

Chondrosarcoma

Fibrosarcoma

Hemangiosarcoma

Other sarcomas

4: Carcinosarcoma—Malignant Mixed Mammary Tumor

5: Benign Neoplasms

Adenoma–simple

Intraductal papillary adenoma (duct papilloma)

Ductal adenoma (basaloid adenoma)

With squamous differentiation (keratohyaline granules)

Fibroadenoma

Myoepithelioma

Complex adenoma (adenomyoepithelioma)

Benign mixed tumor

6: Hyperplasia/Dysplasia

Duct ectasia

Lobular hyperplasia (adenosis)

Regular

With secretory activity (lactational)

With fibrosis–interlobular fibrous connective tissue

With atypia

Epitheliosis

Papillomatosis

Fibroadenomatous change

Gynecomastia

7: Neoplasms of the Nipple

Adenoma

Carcinoma

Carcinoma with epidermal infiltration (Paget-like disease)

8: Hyperplasia/Dysplasia of the Nipple

Melanosis of the skin of the nipple

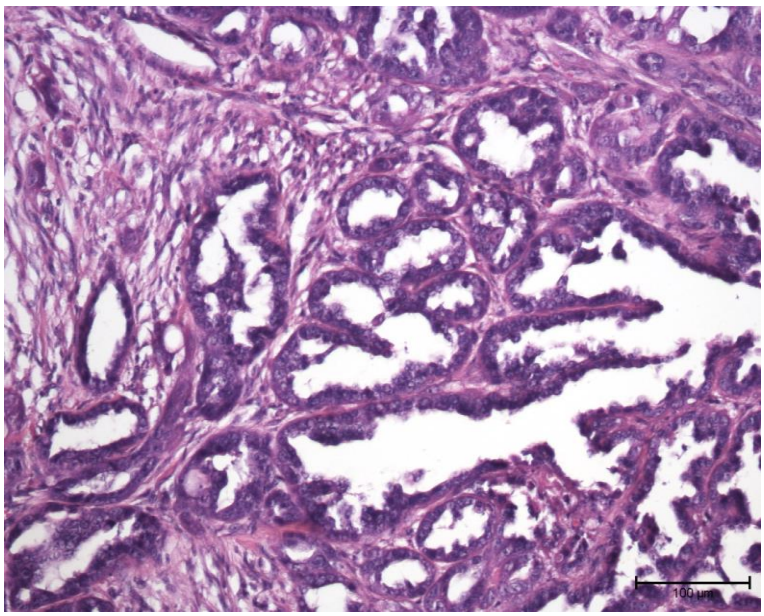


Fig. 7: Simple carcinoma

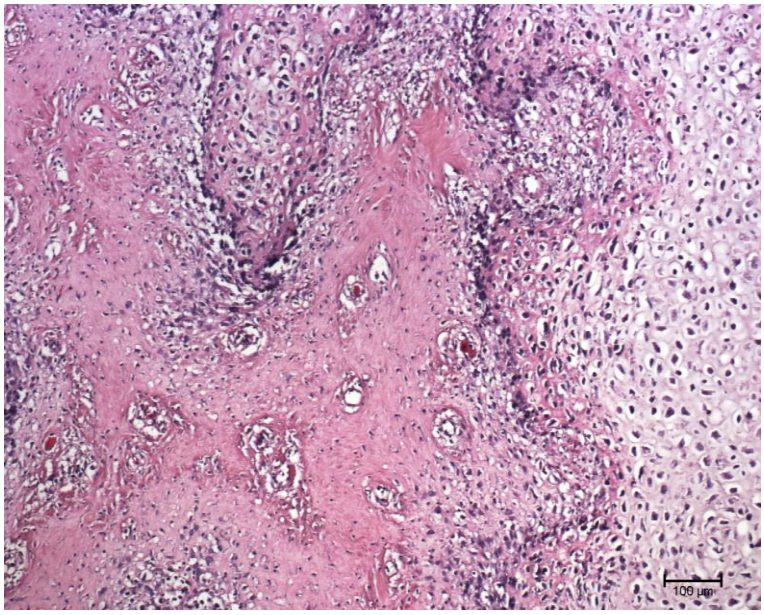


Fig. 8 Mixed carcinoma

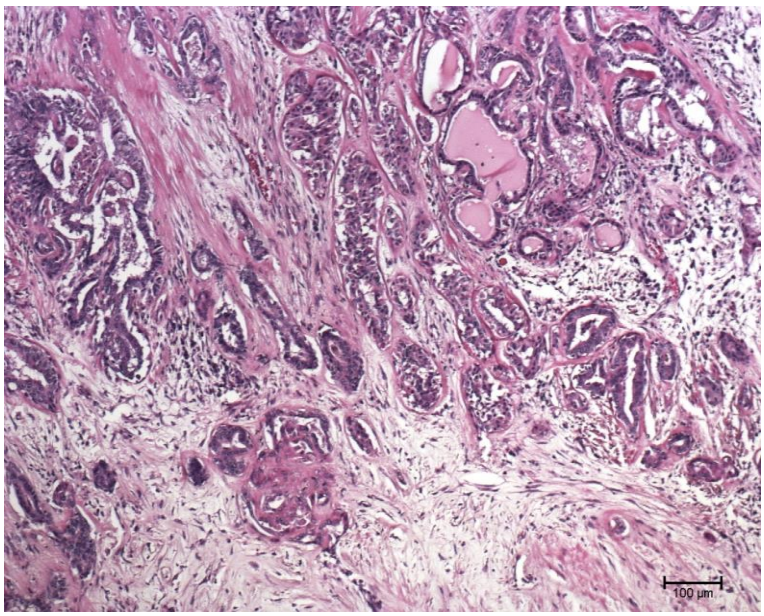


Fig. 9 Complex Carcinoma

Hormonal exposure

As previously mentioned the hormonal expression has been identified to play an important role: bitches spayed before the first estrus have only a 0.5% of risk to develop mammary tumours. The risk increase importantly following the second estrus (Vail and MacEwen, 2000).

In both human and pets is demonstrate as the hormonal exposure plays an important role especially in the epithelial part. In humans the estrogen expression, found in a 20-25% of cases, is associated to aggressive behavior and poor prognosis (Telli et Sledge, 2015). In veterinary studies, important discrepancies were found about these data: the majority of larger and undifferentiated CMTs are less likely to express hormonal receptors although the immunohistochemistry methods of evaluation vary from studies and are not standardized. Due these controversial results is difficult to consider hormonal therapies in veterinary as useful treatment (Ressel et al., 2013).

Therapy: Surgery and Chemotherapy

The surgical approach is the treatment of choice, if possible. Different studies tried to define the correct “dose” of surgical excision: lumpectomy, mastectomy, regional/chain mastectomy or bilateral mastectomies. Actually, the decision need to consider all the previously described factors and is still

subjective. In general is recommended a surgical excision in order to completely remove the tumour and in case of different mammary masses regional or chain mastectomy it is suggested. The surgical excision is questionable in case of inflammatory carcinoma due the diffuse microscopic extension of the disease and for the high grade of early metastasis (Vail and MacEwen, 2000).

Instead, few studies had investigate the systemic chemotherapy and its efficacy has not been confirmed; despite the use is recommended in neoplasm considered at risk for metastasis, recurrence or in advanced disease. As previously described, the hormonal therapy, frequently used in human, not found application in the veterinary field at that moment for the lack of information about their molecular expression and the cost of the medications. The chemotherapic drugs traditionally used in dogs are 5-fluorouracil and cyclophosphamide (Karayannopoulou et al., 2001), doxorubicine and docetaxel (Simon et al., 2006) and gemcitabine (Marconato et al., 2008) but all these studies did not establish post-operative benefit in survival time. Normally the tumoural response to chemotherapy is evaluated according the RECIST criteria (Nguyen et al., 2015).

RECIST criteria

Partial Response (PR) >30% of tumoural reduction in size

Stable disease (SD) any change in size or a reduction < 30% or > 20%

Progressive disease (PD) >20% of tumoural increase in size

According with all these unsatisfactory data, new molecular studies are actually on course with the aim of obtain information about receptors expression and their prognostic and therapeutic relevance.

TYROSINE KINASES RECEPTORS

Tyrosine kinase receptors (TKRs) are an important group of transmembrane receptors that regulate several cellular processes as growth and differentiation (Fig. 10). Most of these receptors are present as monomers on the cell surface and dimerize after the specific binding with growth factor. The tyrosine residues present in the catalytic intracellular domain of the receptor, after this interaction, are phosphorylated and mediate several intracellular pathways.(Fig. 11). In several human and veterinary cancers, the TKRs dysregulation (over expression, mutation or chromosomal translocation) lead to a constitutive phosphorylation of the receptor able to induce uncontrolled signaling involved in cellular survival and proliferation, angiogenesis/metastatisation, and chemotherapy/radiotherapy resistance. For this important role in cancer progression, in the past decade different studies focused on the development of specific drugs able to inhibit the activity of these receptors (Fig. 12).

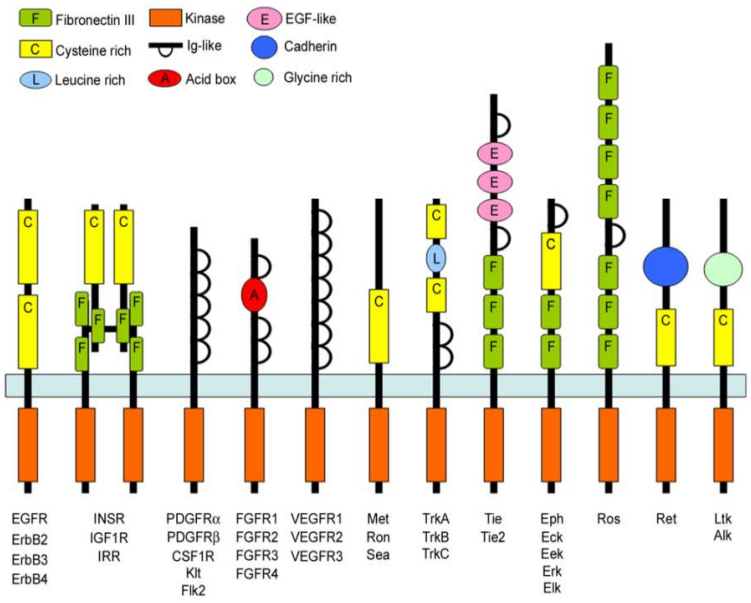


Fig. 10: Different TKRs expressed on the membrane surface.

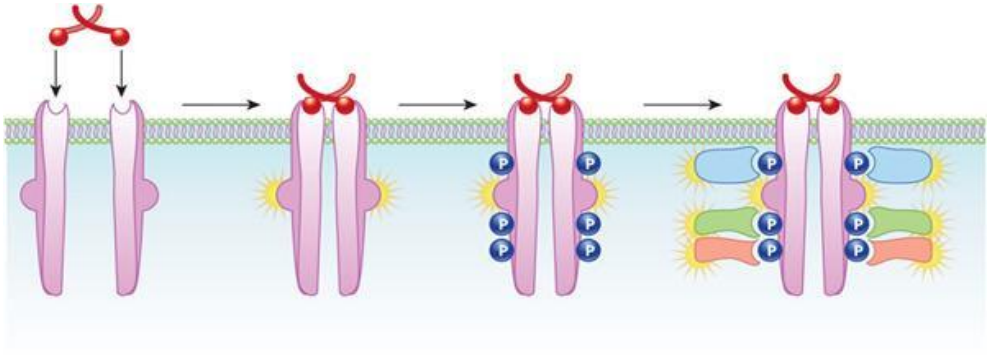


Fig. 11: Molecular Mechanism of activation of RTKs (2010 Nature Education, All rights reserved).

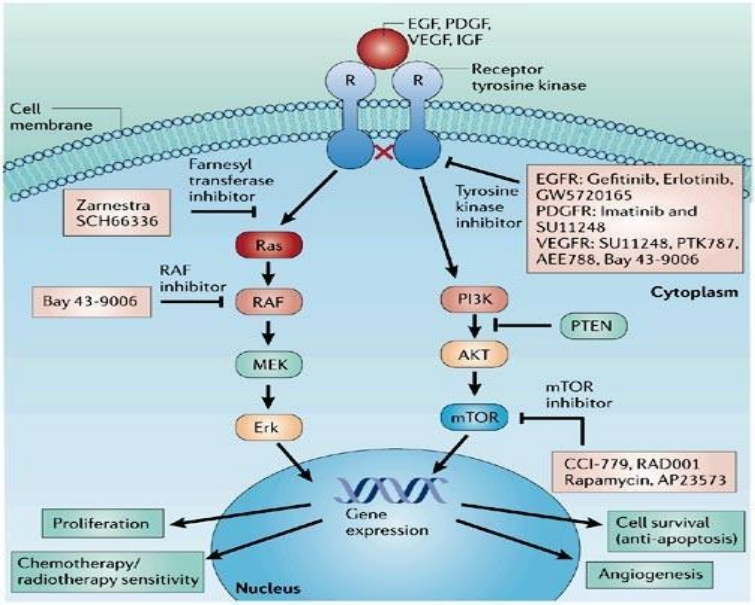


Fig 12: Schematic views of the main pathways activated by the TKRs receptors (http://immugeek.com/four-distinct-subtypes-gastric-cancer-found/).

Platelet-derived growth factor receptors (PDGFR)-Alpha and Beta, vascular endothelial growth factor receptor-2 (VEGFR-2) and CD117, belong to tyrosine kinases receptors (TKRs). Against these receptors have been developed specific drugs as Imatinib for the human GIST (Gastrointestinal Stromal Tumors) and Masitinib phosphate or Toceranib phosphate in canine mast cell tumours (London et al., 2003; Grant et al., 2016; Mc Donnel et al., 2017).

PDGFR-Alpha and Beta are activated by PDGFs, VEGFR2 by VEGFs factors while CD117 by SCF (Stem Cell Factor) soluble factors. These receptors, after specific binding, are able to activate many of the major cellular signal transduction pathways (van Biesen et al., 1995; Liu et al., 2011; Nikolaienko et al., 2012; Criscitiello et al., 2014; Zhu and Zhou, 2015). Moreover they are involved in physiological processes, as inflammation and tissue regeneration, as well in tumoural diseases (Alvarez et al., 2006; Demoulin and Essaghir, 2014; Demicco et al., 2015; Toska and Baselga, 2016). In particular, in cancer disease PDGFR alpha and beta regulate cell growth and division, activating many of the same major signal transduction pathways involving PI3K/AKT pathways, RAS and MAPK (Fig. 13).

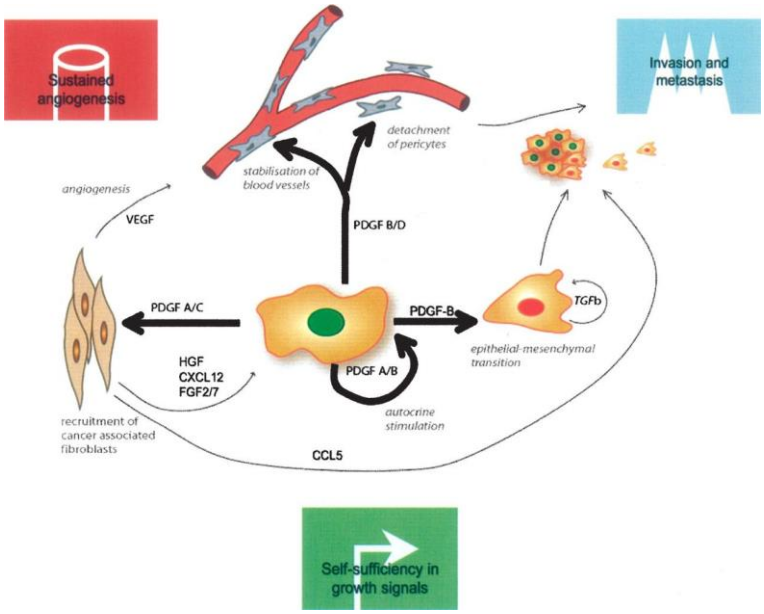


Fig. 13: different activated pathways and their role in the organism (Andrae et al., 2008,).

VEGFR2 is involved mainly in the angiogenesis and tumoral invasion and metastasis and activated by vascular endothelial growth factor (VEGFs) that are angiogenic factor released by neoplastic and stroma cells (Fig.14);

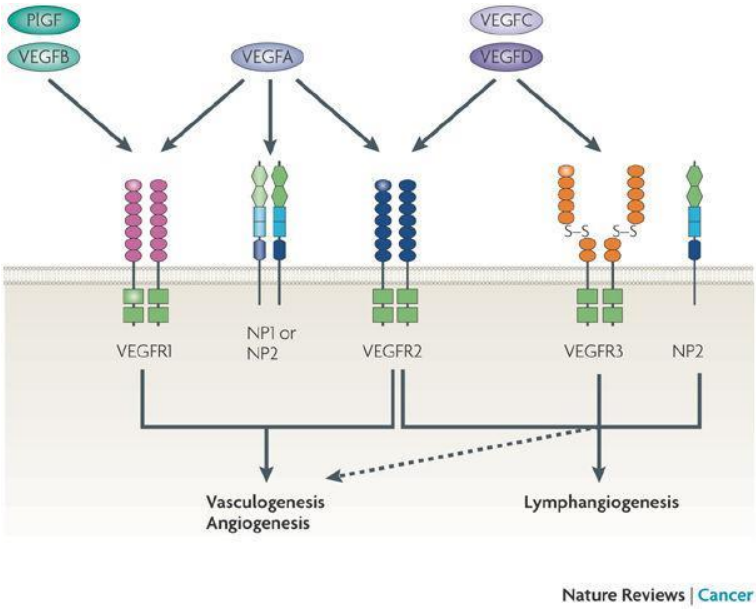


Fig. 14: VEGFs stimulation of the TKRs on the membrane surface (Ellis et al., 2008).

CD117 is a mainly involved in mast cell growth, differentiation and chemotaxis and is able to activate MAPK kinase, PI3K/p-AKT and STAT pathways (Fig. 15).

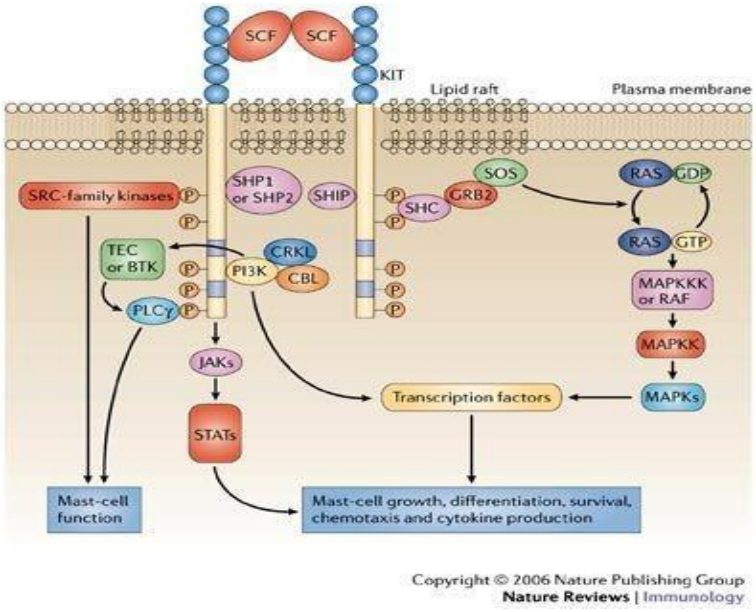


Fig. 15: CD117 (c-Kit) intra-citoplasmatic pathway.

PDGFRα, PDGFRβ, VEGFR2 and CD117 in Canine Tumors

PDGFR alpha, PDGFR beta

In the last years, numerous veterinary studies demonstrated the over-expression or activation of PDGFRs in several tumours as osteosarcoma, lymphoma, thyroid and apocrine gland carcinoma, melanomas and hemangiosarcoma (Urie et al., 2012; Maniscalco et al., 2013; Aricò et al., 2014; Abou Asa et al., 2015; Iussich et al., 2017). In canine osteosarcomas, PDGFRα and PDGFRβ were expressed in 78% and 81% of cases, respectively suggesting an autocrine and/or paracrine loop, an important role in the etiology in this neoplasm and maybe suitable targets for the treatment with a specific TKI (Maniscalco et al., 2013).

The identification of PDGF and its receptors in aggressive canine T-cell lymphomas, suggested their involvement in the pathogenesis and implies a functional autocrine and/or paracrine loop of growth stimulation, as in osteosarcoma. Even if autocrine receptor activation may occur in T-cell lymphomas, is still not known if this is a critical or a contributing event in its development (Aricò et al., 2014). Moreover, the structural aberrations of PDGFRs, founded in different lymphoma's cases, that lead to overexpression or expression of abnormal proteins have been also described in canine vascular tumours (*Abou Asa* et al*., 2013*) and in both these studies the authors suggest the need of more prospective studies to understand whether PDGFs and PDGFRs may represent new therapeutic targets.

VEGFR2 in Canine Tumors

In the study of Urie et al., positive immunoreactivity for VEGFR2 was noted in the cytoplasm of tumor cells in 79% of primary and 60% of metastatic canine anal sac carcinomas and in 40% of thyroid carcinomas samples. Strong cytoplasmic and stromal staining for PDGFRα was reported in all primary and metastatic anal sac carcinomas and all of the thyroid carcinomas samples. Moreover, PDGFRβ intense stromal staining was noted in all tumor samples. These data suggested that while these RTKs
may be present in most anal sac carcinomas and in thyroid carcinomas, most probably they do not exist in a state of continual activation/signaling observed in the setting of typical RTK dysregulation associated with mutation, chromosomal translocation, and over-expression. For these reasons, the

authors did not clearly directly correlate these expressions with a causative role in tumor growth and survival but their significant presence in the stromal part suggested an important role on the vascular endothelium and tumoural microenvironment and a possible target at this level for the TKIs use (Urie et al., 2012).

In canine oral melanoma finally, Iussich et al. founded a PDGFRs co-expression in 37.5% of cases and that a positivity for PDGFR-α and -β receptor was present in 54.2 and 47.9% of cases, respectively. The statistical analysis performed in this study showed that PDGFRs co-expression and Ki67 proliferative values >19.5% were both associated with worse prognosis. For these reasons, the authors suggested a role in the pathogenesis and progression of canine oral melanoma and the PDGFR α and β co-expression association with a worse prognosis (Iussich et al., 2017).

CD117 in Canine Tumors

CD117, results mainly over-expressed and/or mutated in canine mast cell tumours but it also over-expressed in canine lymphomas and oral melanomas (Newman et al., 2012; Giantin et al., 2013; Patruno et al., 2014). In canine mast cell tumours this receptor has been largely studied and its aberrant expression is correlated to increase angiogenesis, and higher histopathological grade. Moreover, Patruno et al. underlined as the inhibition of activated mast cells, important for the tumoral angiogenesis, with TKIs could be an important result in the comparative oncology (Patruno et al., 2014).

In canine melanomas, Newman et al. demonstrated that CD117 expression is expressed in the 44.3% of the submucosal neoplasm and in the 27.9% in the junctional neoplastic melanocytes; its altered expression was correlated with prognosis (Newman et al., 2012).

Finally, Giantin et al. specifically evaluated CD117 in T-cell lymphoma demonstrating its higher expression in grade T-cell lymphomas suggesting the use of specific TKI in future clinical trial was suggest (Giantin et al., 2013).

In canine mammary tumours, in the study of Brunetti el al. (2014) no relationship between tumour histotype and the presence or absence of CD117 expression was seen. The results showed a decrease in CD117 expression in benign tumours compared with normal tissue, but not in malignant types, as reported in human breast cancer. The authors explained this result due the extreme heterogeneity of the canine neoplasms.

Brunetti et al. noted a significant correlation between CD117 labelling pattern and the histotype of malignant forms, with an increasing percentage of cytoplasmic-only labelling from the well differentiated to the less-differentiated histotype.

The Ki67 proliferative activity, evaluated in their study, was associated with the three CD117 labelling parameters in malignant tissue, suggesting a link between the presence of the receptor and proliferative activity. Brunetti el al. concluded suggesting as, due the correlation between CD117 expression and proliferative activity, TKI may have a role in the therapy of CMTs.

RATIONALE OF THE STUDY AND AIM OF THE WORK

In the last years, an increasing interest was placed in comparative oncology about the tumoural microenvironment and the possible control of angiogenesis as method to decrease neoplastic progression and metastatisation. In the veterinary field, vascular endothelial growth factor and its receptors (VEGF and VEGFRs) expression were largely examined in canine haemangiosarcomas, lymphomas and cutaneous squamous cell carcinomas. (Yonemaru el al., 2006; Al-Dissi et al., 2007; Wolfesberger et al., 2007; Sabattini et al., 2009; Martano et al., 2016; Millanta et al., 2016).

Yonemaru and Sabattini indicated in their studies the presence of an autocrine or paracrine of VEGF pathway in the proliferation of canine haemangiosarcomas. In these studies, the authors demonstrated that VEGF is secreted from neoplastic cells, binds its receptor on the neoplastic endothelial cells and promotes their proliferation (Yonemaru el al., 2006; Sabattini et al., 2009). The results obtained by Yonemaru and colleagues suggested that the expression of both the growth factor and receptor, especially flk-1, might be associated with the malignant proliferation of the cells and may also provide a theoretic explanation for the success of the inhibitory activities of VEGF in tumor therapy.

The same autocrine pathway for VEGF probably operates in canine cutaneous squamous cell carcinomas where VEGF and VEGFR-2 expression was associated to cell proliferation (Al-Dissi et al., 2007).

Wolfesberger et al. evaluated VEGF and VEGFRs in canine different type of lymphomas and founded that VEGF protein was expressed by 60% of the tumours with diffuse cytoplasmic labelling of the neoplastic cells. Unfortunately, they reported, as most neoplasms did not express VEGFR-2 but in 7% of sections, there was focal labelling of neoplastic and endothelial cells, with a cytoplasmic and perinuclear pattern. Due their observed variability in expression of VEGF and its receptors, related to the fact that lymphoma is a heterogeneous lymphoproliferative tumour, the authors suggested for the first time the utility of evaluated an individual approach in the clinical treatments (Wolfesberger et al., 2007).

In the case of the canine oral squamous cell carcinomas, Martano et al. reported that VEGF and the proliferating cell nuclear antigen expression increased significantly between normal oral tissue and neoplastic tissue, and between well and moderately/poorly differentiated tumours. In this study, VEGF expression was strongly correlated with microvessel density and the authors concluded that VEGF may promote angiogenesis stimulating endothelial cell proliferation and may induce tumoural cell

proliferation, suggesting the evaluation of this factor as useful additional criterion for estimating malignancy and growth potential in canine oral squamous carcinomas (Martano et al., 2016).

Finally Millanta et al. studied VEGFs/VEGFRs in feline and canine mammary carcinomas by the immunohistochemical assessment of VEGF expression and micro vessel density (MVD) quantification. Millanta reported as an increased VEGF expression was not correlated with clinicopathological parameters or a poorer prognosis; while MVD was found to be significantly correlated to the histologic type, tumour grading, and to the overall survival. The authors suggest the VEGF/KDR role in malignant transformation and tumor progression (Millanta et al., 2016).

In human breast cancer, PDGFRs, VEGFR2 and CD117 are been showed involved in both cellular differentiation and invasion as well as in tumoural microenvironment where they found an important role in metastatisation and angiogenesis (Das Roy et al., 2013; Olgasi et al., 2014; Paulsson and Micke, 2014; Pinto et al., 2014; Son et al., 2014). These authors focused on the role of these receptors in tumoural microenvironment and on the role of mast cells, regulatory T cells (T-reg) and tumour infiltrating inflammatory cells, with these receptors. (Das Roy et al., 2013; Olgasi et al., 2014; Son et al., 2014). Regarding TKRs in details Pinto et al. provided evidence that the PDGF signaling pathway leading to estrogen-independent proliferation and angiogenesis can target luminal cancer cells, and speculate that stroma-directed therapies, including anti-PDGFR agents like Imatinib, may be useful in combination with other therapies for treatment of luminal cancers (Pinto et al., 2014). Unfortunately no a clear association between TKRs expression and survival was found (Paulsson et Micke, 2014)

For this reason, the potential use of targeted therapy found importance in the control of the tumour but also in the neo-vascularization and in the control of present/possible distant metastasis (Simon et al., 2004; Zhu et al., 2014, Zhu and Zhou, 2015). For example, Simon et al. reported as despite CD117 is not frequently mutated in breast cancer, some type of neoplasms as medullary breast cancers could strongly benefit of a CD117 target therapy (Simon et al., 2004). A different author (Zhu et al., 2014) reported instead a frequent expression in triple negative breast cancer suggesting in few cases the benefit of a TKI.

Zhu et al. demonstrated in breast cancer an interaction between VEGFR-2 overexpression and mutated p53 cells and with the JCK2/STAT3 pathway suggesting the utility of a VEGFR-2 inhibitor in breast cancer therapy, in particularly against cancer stem cells.

To our knowledge, there is still no data regarding the expression of PDGFRs in CMTs; while preliminary results on VEGFR-2 and CD117 expression reported that VEGFR-2 is expressed in 58% of CMTs and statistically associated with VEGF immunoreactivity in cancer cells. Moreover, CD117 is reported to be highly expressed in malignant forms suggesting an important role of these receptors in inflammatory and high-grade canine mammary carcinomas (Kubo et al., 1998; Restucci et al., 2004; Millanta et al., 2006; Millanta et al., 2010; Brunetti et al., 2014; Santos et al., 2014, Carvalho et al., 2015).

The use of specific inhibitors for Tyrosine kinases receptors (TKIs) in human and veterinary medicine have rapidly expanded in the last years with the hope to find drugs targeting directly the tumour and reducing the systemic toxicity. Small molecule inhibitors are medications that work primarily blocking the ATP-binding site of kinases, generally acting as competitive inhibitors. In absence of phosphorylation, the receptor is unable to activate the intra-cellular pathways interrupting the survival/growth signal, essential for the tumour survival.

Their use in human clearly depends on receptor expression, activation and/or mutation, which are usually evaluated in tissues before treatment (Liao et al., 2002; Roskoski, 2014; Yadav et al., 2014; Telli and Sledge, 2015). The same approach is suggested for canine mast cell tumours, in which particularly in case of masitinib's therapy, the mutation of the receptor c-Kit need to be evaluated in quantitative PCR before the treatment (Downing et al., 2002; Amagai et al., 2013; Gil da Costa et al., 2015), but not clear data are actually available for other oncological conditions.

The TKRs evaluated in this study represent an attractive target for toceranib phosphate (Palladia, Zoetis), a veterinary TKI which selectively inhibits VEGFR-2, PDGFRs and CD117 and which is currently used for different neoplasms, including mast cell tumours, anal sac and nasal carcinomas, metastatic osteosarcoma, head and neck and thyroid carcinomas (London et al., 2003, 2012, 2015; Gardner et al., 2015). The attractive of using this target therapy is represented by a more specific action on these very aggressive tumours, with the hope to reduce the chemotherapic resistance and the antiblastic collateral toxicity on the gastrointestinal tract and on the bone marrow.

Based on the previous considerations, the aims of the present study are to characterize the expression of PDGFRα, PDGFRβ, VEGFR-2 and CD117 in canine mammary carcinomas analyzing the immunohistochemical and the molecular expression, in order to provide information regarding TKRs as potential prognostic biomarkers and molecular target in this tumour. Moreover, this study would provide preliminary results about the in vitro and in vivo effect of toceranib phosphate (Palladia, Zoetis).

MATHERIALS AND METHODS

Histological Sample collection

Eighty-three spontaneous malignant CMTs, 16 benign CMTs, 4 normal and 7 hyperplastic mammary surgical samples were retrieved from the archive of the University of Turin (Italy) and from the Hospital of the Department of Veterinary Sciences of the University of Turin (Italy). For malignant tumours, cases were included if initial staging was performed and complete surgical resection was achieved. Data regarding physical examination, tumour size and stage (abdominal ultrasound and three thoracic radiographs) were collected from the medical records. Cases with regional or distant metastases, incomplete surgical resection or treated with chemotherapy were excluded from the study. Depending on the case and on the clinician's discretion mammectomy or radical mastectomy was performed (Horta et al., 2015). Follow up, corresponding to clinical evaluation, abdominal ultrasound and thoracic radiographs, was performed for two years every three months. Animals that died of unrelated causes during the follow up period were excluded from the study. Survival endpoint included disease-free interval (DFI), defined as the number of days between surgery and tumour recurrence and/or evidence of metastatic disease. Where different subtypes of CMT were found in the same dog, the highest histological grade type was selected for analysis.

Clinical Trial

For the clinical trial 38 dogs with not resectable or metastatic mammary carcinomas were enrolled. All this cases presented a single mass, not operable, with a staging complete performed with thoracic radiographs and abdominal ultrasound. The diagnosis was performed histologically or, when not possible, at least cytologically. In case of enlarged regional lymph node, a cytology or histology was performed to confirm the metastatic disease. All the oncological condition were classified according the TNM system.

The toceranib phosphate (Palladia, Zoetis) was administer at 2.75-3.25 mg/kg every other day or with the Monday-Wednesday-Friday regimen. The toxicity was assessed based on the dog's history, physical examination and complete blood exams and in some of these cases, the dose was reduced based on clinician's decision. The overall progression free interval (PFI) was consider as the number

of days from diagnosis to tumoural progression. Partial response (PR), stable disease (SD) and progressive disease (PD) were defined according the RECIST criteria.

The results were evaluated according preliminary results obtained by London (London et al., 2003) in which was defined as positive response a PR or SD more than 10 weeks.

Histological diagnosis and Immunohistochemical analysis

Samples from the archive of the University of Turin and from clinical trial were fixed in 10.0% buffered formalin solution for at least 24 h, embedded in paraffin wax blocks, cut at 4-µm thick slices and stained with haematoxylin and eosin (HE). Sections were histologically classified according to Goldschmidt et al. classification (2011). Immunohistochemical (IHC) analysis were carried out on 4-µm formalin-fixed and paraffin-embedded sections. Primary antibodies (Table 1) were detected using the avidin-biotin peroxidase complex technique with the Vectastain Elite ABC Kit (Vector Laboratoires). Canine skin, prostatic carcinoma, normal spleen and mast cell tumour were used as external positive controls for 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 Biothecnology). Cytoplasmic immunopositivity was evaluated separately in both epithelial and stromal (fibroconnective tissue that forms bundles within and surrounding the tumour) compartments.

PDGFRs and VEGFR-2 immunopositivity was evaluated observing 10 high-power fields (400x) randomly selected and considering the number of positive cells and the intensity of the positivity, according to the semi-quantitative scoring method adopted by Donnem et al. (2008).

In this method, the cell density was scored as 1 = low density; 2 = intermediate density; 3 = high density. The dominant staining intensity in both tumor and stromal cells was scored as 0 = negative; 1 = weak; 2 = intermediate; 3 = strong. The proportion score and intensity score were then added to obtain a total score considering a total score ≥ 3 as positive.

CD117 immunoreactivity was evaluated considering the presence/absence and the percentage of positive cells as follows: 0 for negative, 1 for focal (1-19.0% of labelled cells), 2 for intermediate (20-49.0%), and 3 for diffuse (>50.0%) (Brunetti et al., 2014).

Immunolabeled slides were masked for blinded examination independently scored by two observers (S.I., L.M.). In case of disagreement, a consensus was reached using a multi-head microscope.

Canine mammary carcinoma (CMC) cell lines

The University Of Veterinary Medicine Of Tokyo (Japan) provided two canine primary (CYPp, CTB) and one metastatic (CYPm) mammary carcinoma cell lines, while the American Type Culture Collection (ATCC) provided CF33 cell line. Final small letters such as “p” and “m” indicate respectively cell lines derived from primary and metastatic lesions. All cell lines were cultured in RPMI standard medium supplemented with 10% FBS (fetal bovine serum), 1% glutamine, 100 □g/mL penicillin and 100 □g/mL streptomycin. Only for CF33 DMEM medium supplemented by the same reagents was used (Murai et al., 2012).

Establishement of Chicca, Lilly, Milly and NEM cell lines

Tissue samples from three spontaneous grade III simple carcinoma surgically treated dogs (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 then centrifuged and suspended in Dulbecco’s modified Eagle medium. For “Lilly” cell line, 10 □g/mL insulin (Sigma-Aldrich) was added. NEM cell line,established according to method present in literature (Sánchez-Céspedes et al., 2013), were grown in Dulbecco’s modified Eagle’s Medium/Nutrient Mixture F12 Ham (DMEM/F12; Sigma–Aldrich) supplemented with 5% fetal calf serum (FCS), 5000 IU/mL penicillin and 5 mg/mL streptomycin, 10 □g/mL insulin, hydrocortisone 0.5 □g/mL and choler toxin 10 ng/mL. In order to confirm epithelial cells 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 protein lysate obtained from normal and CMCs. Total proteins were extracted in boiling lysis buffer containing 1% SDS and 0.1% M Tris-HCl (pH 6.8). Total proteins were quantified by Lowry method and 20 µg were separated on 8% SDS- polyacrylamide gel (PAGE). After transferring into Hybond-C Extra membranes (American Biosciences), these membranes were blocked at room temperature for 2 h with Tris-buffered saline (TBS, 10 m*M* Tris and 150 m*M* NaCl, pH 7.4) containing 10% BSA and then incubated overnight at 4°C with the primary antibodies reported in Table 1. After washing in TBS-Tween (0.5%), membranes were incubated with

secondary horseradish peroxidase (HRP)-linked antibody diluted 1:1000 in PBS-Tween for 1 h. Finally, the membranes washed in TBS-Tween for 30 min, were incubated in enhanced chemiluminescence reagents (Super Signal West Pico Mouse IgG Detection Kit, Thermo Scientific).

Antibody	Type	Source	IHC	WB
VEGFR-2	Rabbit polyclonal	Santa Cruz Biotechnology	1:150	1:1000
PDGFRα	Rabbit polyclonal	Cell Signaling Technology	1:100	1:1000
PDGFRβ	Rabbit polyclonal	Santa Cruz Biotechnology	1:200	1:1000
PDGFRβ	Rabbit polyclonal	Cell Signaling Technology	1:200	1:1000
CD117 (c-Kit)	Rabbit polyclonal	Dako, Glostrup, Denmark	1:400	1:1000

Table 1. Sources and dilutions of the antibodies employed in immunohistochemistry (IHC) and Western Blot (WB) techniques for platelet-derived growth factor receptors (PDGFR)-Alpha and Beta, vascular endothelial growth factor receptor-2 (VEGFR-2) and CD117.

Immunocytochemistry

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

Total RNA extraction and quantitative PCR expression analysis

Total RNA was extracted using TRIZOL Reagent (Sigma-Aldrich) and cDNA was synthesised from 1 µg of total RNA using the QuantiTect Reverse Transcription kit (Qiagen) including genomic DNA removal reagent. cDNA was subjected to quantitative PCR using the IQ SYBR Green Supermix (BioRad) and the IQ5 detection system (BioRad). Primer sequences are listed in Table 2. A relative quantification assay corresponding to the comparative cycle threshold (Ct) method was used. The amount of target, normalized to the endogenous housekeeping gene (glyceraldehyde-3-phosphate

dehydrogenase, GAPDH) and relative to the calibrator (control sample), was transformed by 2ΔΔCt (fold increase), where ΔΔCt= ΔCt (sample) - ΔCt (control). ΔCt is the Ct of the target gene subtracted from the Ct of the housekeeping gene. NEM cell lines were used as control.

Primer	Gene	Sequence
Forward	VEGFR-2	5'-CATGCACGGTCTACGCCGTCC-3'
Reverse	VEGFR-2	5'-CAGCTTGGGCGGGCTTGTAGG-3'
Forward	CD117	5'-CTCGCGGCGCCTGGGATTTT-3'
Reverse	CD117	5'-GAAGAGCCTGTCCGGACGCC-3'
Forward	PDGFRα	5'-CATCCCCCTGCCCGACATCG-3'
Reverse	PDGFRα	5'- TGAGCTGTGTCTGTTCCTCTTGCC-3'
Forward	PDGFRβ	5'-GTCCTCAAAGGCCAGGCACTGTGG-3'
Reverse	PDGFRβ	5'-CCCCGGGGGTGTGATGACCAG-3'
Forward	GAPDH	5'-GGCACAGTCAAGGCTGAGAAC-3'
Reverse	GAPDH	5'-CCAGCATCACCCCATTTGAT-3'

Table 2. Primer sequences used in q-PCR.

Proliferation assay after inhibition in vitro with toceranib phosphate

Based on the data obtained, CYPm and CTB cell lines, overexpressing PDGFRα 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 et al., 2013). Ten thousand cells from each cell line were seeded in 96-well plates, allowed to attach overnight and were treated with different concentrations of toceranib (150 nM, 300 nM, 600 nM, 1 μM, 1.5 μM) diluted in Dymethyl sulfoxide 10%, for 12, 24 and 48 h. In order 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 three times in triplicate.

Statistical analysis

IHC results and clinic-pathological findings were grouped into contingency tables and analyzed using Fisher's exact test or χ^2 test. Survival plots were computed using the Kaplan–Meier method and tests for survival differences were performed using the log-rank test. The Cox proportional hazard model for multivariate analysis was used to study the effects of different survival variables. Parameters correlated with prognosis were evaluated in interaction using a Cox proportional hazard regression model. q-PCR and proliferating data were analyzed using Student's t test. Data were analyzed with MedCalc Statistical Software version 13.3 (MedCalc Software bvba). P <0.05 was considered statistically significant.

RESULTS

Clinical data

Eighty-three CMTs surgically removed from 83 bitches (mean age: 10.36 ± 2.4 years) were evaluated. Tumour size was less than 3 cm, between 3 and 5 cm and more than 5 cm in 51.8%, 26.5% and 21.7% of the cases, respectively.

Sixty-four of the 83 dogs (77.1%) survived more than 700 days from surgery and 19 dogs (22.9%) died due to causes related to the mammary carcinoma during the follow up period. Among deceased animals, 31.0% had tumoural recurrence and 69.0% died for distant metastasis (68.4% of the cases in the first 6 months, 21.0% between 6 and 12 months and 10.5% between 12 and 24 months).

Histopathology

According to the WHO classification, 50.6% of cases were histologically classified as simple carcinoma while 30.1% and 19.3% as complex and mixed types, respectively. Twenty-four carcinomas (28.9%) were histologically classified as grade I, 34 (41.0%) as grade II and 25 (30.1%) as grade III. Cell lines collected were histological classified as simple carcinomas (Chicca, Lilly, CYPp, CTB, Milly and CF33).

IHC analysis

IHC results and staining are summarized in Table 3A-B and Fig. 16, respectively. 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.

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	

Table 3A. Relationships between tumour characteristics and Tyrosine Kinase Receptors expressions in neoplastic cells (Gattino et al., 2017).

	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	

Table 3B. Relationships between tumour characteristics and Tyrosine Kinase Receptors expressions in stromal cells (Gattino et al., 2017)

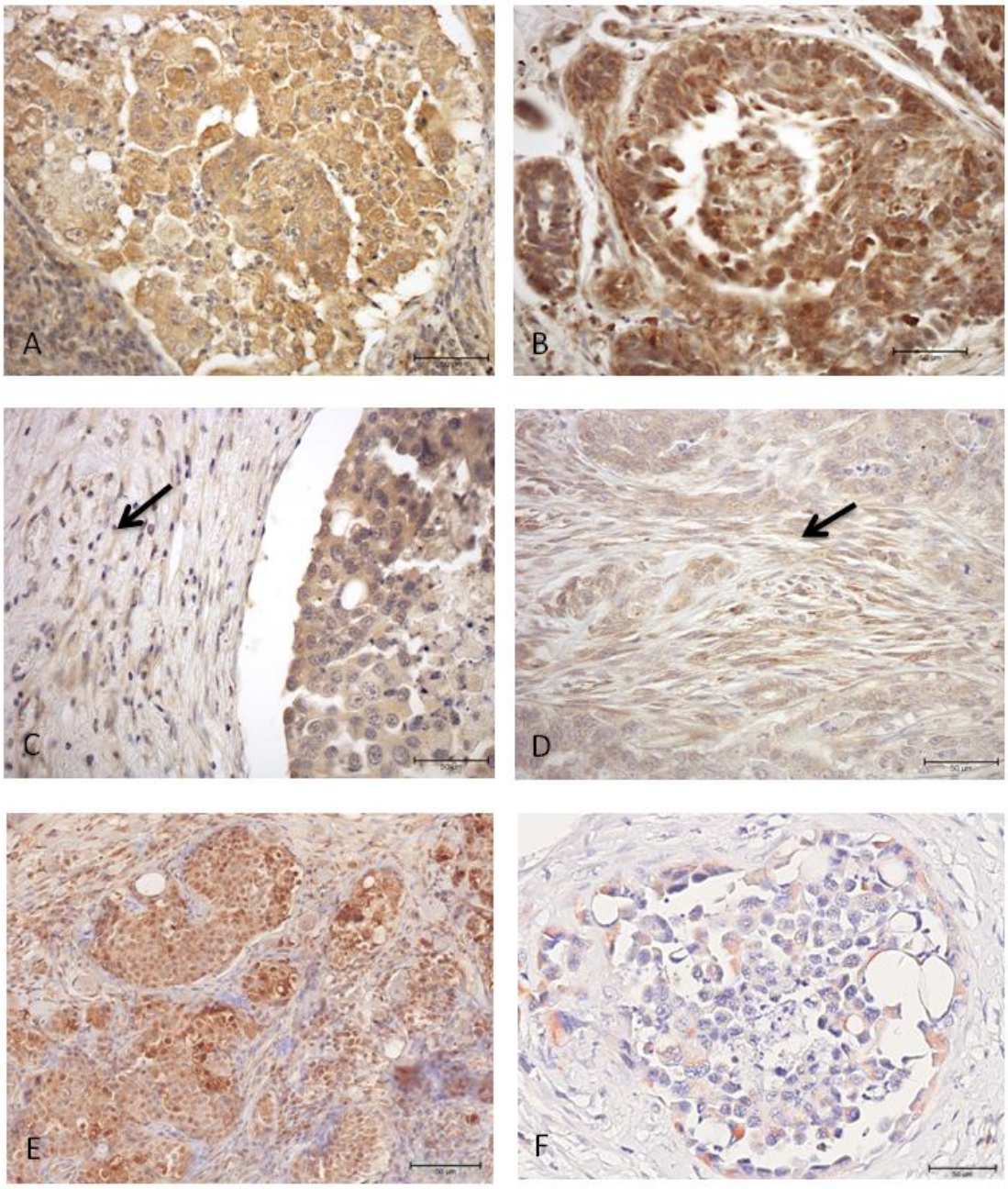


Fig. 16. Simple carcinomas. Picture A and B showed a strong cytoplasmic immunolabelling in the neoplastic cells for platelet-derived growth factor receptors (PDGFR)-α and β, respectively. Pictures C and D showed intermediate immunolabelling in the stromal cells for PDGFRα and β, respectively. Picture 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 (Gattino et al., 2017).

Regarding the PDGFRs receptors, tumoural cells showed a significant lower immunolabelling for PDGFRα and PDGFRβ (*P* <0.05) in simple carcinomas compared to complex and mixed tumours.

Moreover, in the stromal compartment, PDGFRβ showed a significant lower immunolabelling in simple carcinomas compared to complex and mixed carcinomas (*P* <0.05). PDGFRs expression was not statistically correlated with prognosis, despite negative patients having worse prognosis (Fig. 17 and 18).

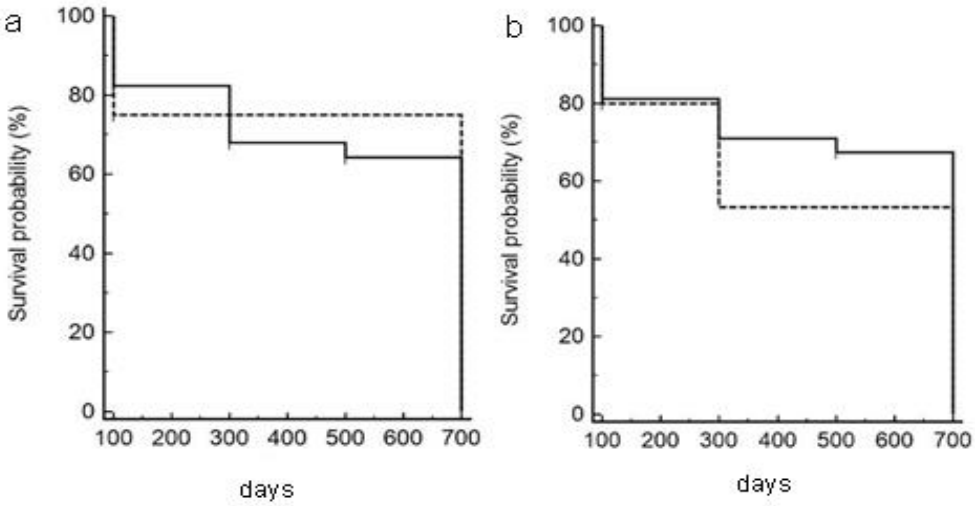


Fig. 17: (a) Kaplan–Meier curve of DFI in bitches with simple mammary carcinoma and platelet-derived growth factor receptors (PDGFR)-Alpha negative (continuous line; median DPI: 700 days) and positive (dotted line; median 700 days) in stroma (Log-rank test: P > 0.05). (b) Kaplan–Meier curve of DFI in bitches with simple mammary carcinoma and platelet-derived growth factor receptors Beta negative (continuous line; median DPI: 700 days) and positive (dotted line; median 700 days) in stroma (Log-rank test: P > 0.05).

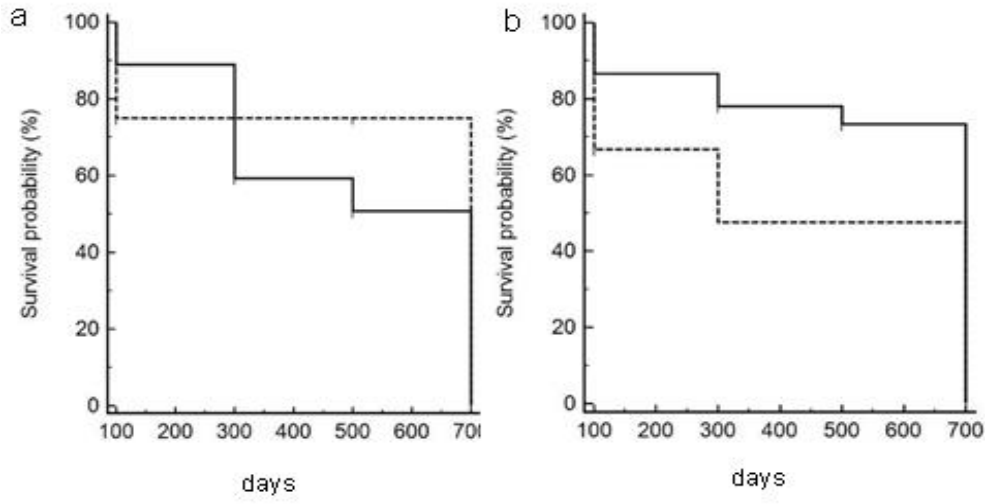


Fig. 18: (a) Kaplan–Meier curve of DFI in bitches with in simple mammary carcinoma PDGFRβ negative (continuous line; median DPI: 700 days) and positive (dotted line; median 700 days) in tumoural cells (Log-rank test: P > 0.05). (b) Kaplan–Meier curve of DFI in bitches with in simple mammary carcinoma PDGFRβ negative (continuous line; median DPI: 700 days) and positive (dotted line; median 300 days) in stroma (Log-rank test: P > 0.05).

When comparing the epithelial component versus stromal cells, VEGFR-2 was expressed in 46/83 (55.4%) and in 18/83 (21.7%) of the malignant tumours, respectively. In the benign type the VEGFR-2 expression was instead 62.5% and 37.5% in epithelial and stromal compartment, respectively. No statistically significant correlation was found between histological grade, histotype and DFI.

CD117 was negative in 61.4% of the cases; positive samples showed focal positivity (28.9%), intermediate pattern (6.1%), or diffuse immunolabelling (3.6%). 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 between CD117 and histological grade.

Molecular Investigations

Specific protein bands corresponding to canine PDGFRα and VEGFR-2 were detected in CF33, CYPp, CYPm and in CTB cell lines respectively, while CD117 and PDGFRβ were not identified by Western Blot analysis (Fig. 19).

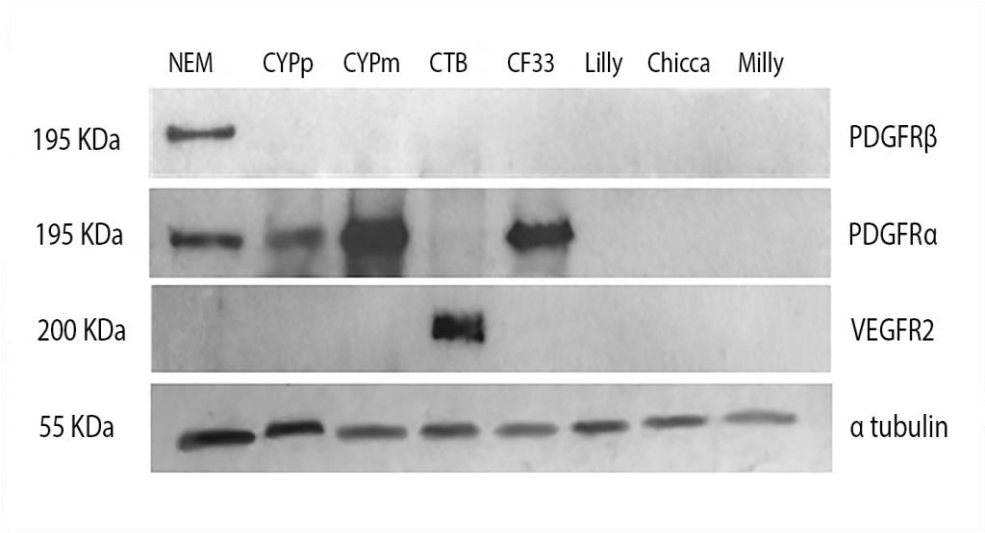


Fig. 18. Western Blot analysis of Platelet-derived growth factor receptors (PDGFR)-α and β and Vascular endothelial growth factor receptor-2 expression in normal epithelial mammary cell lines NEM (lane1), and neoplastic cell lines CYPp (lane 2), CYPm (lane 3), CTB (lane 4), CF33 (lane 5), Lilly (lane 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 (Gattino et al., 2017).

Quantitative PCR results showed that: VEGFR-2 transcript is present in Lilly and CTB cell lines, PDGFRα in CYPp, CYPm and CF33, while PDGFRβ is expressed at low level in all analyzed cell lines. Finally CD117 transcript was not detectable in all analyzed cell lines (Fig. 20).

Statistically significant over-expression of VEGFR-2 was found in CTB and Lilly cell lines. Additionally, PDGFRα was significantly over-expressed in CYPp, CYPm and CF33 cell lines compared to NEM, while a statistical down-regulation of VEGFR2 and PDGFRβ was found in CYPp, CYPm and Chicca cell lines. Milly cell line resulted negative for all analyzed genes.

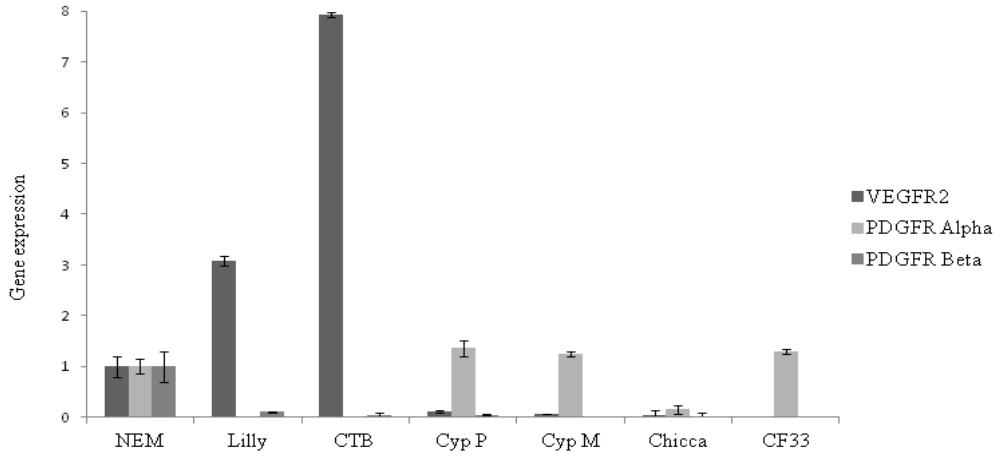


Fig. 20. Expression by quantitative PCR of PDGFRα, PDGFRβ and VEGFR-2 in CMTs cell lines. PDGFRα mRNA was expressed at higher levels in CYPp, CYPm and CF33. PDGFRβ was detected in all the cell lines at a lower level compared to NEM (control). VEGFR-2 was expressed at a higher level in Lilly and CTB cell lines. The increased fold of each specific mRNA was normalised with NEM cell line and the error bars indicate the standard deviation of experimental triplicates.

In order to confirm data obtained from western blot and quantitative PCR, immunocytochemistry against PDGFR alpha, PDGFR Beta, CD117 and VEGFR2 was performed on CMC cell lines as shown in fig. 21).

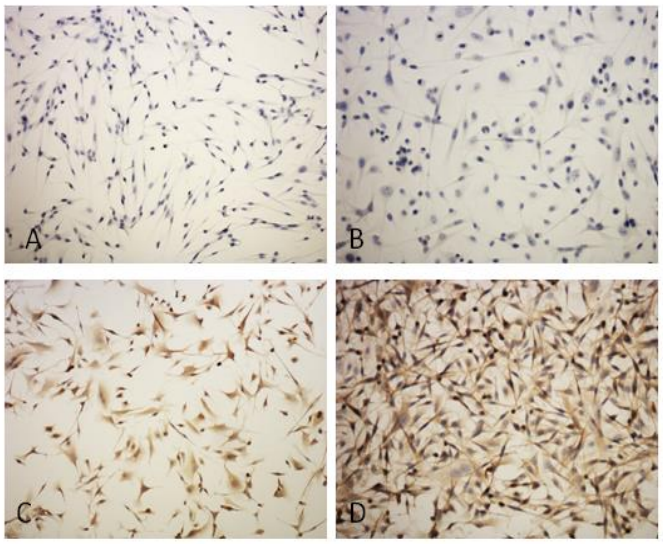
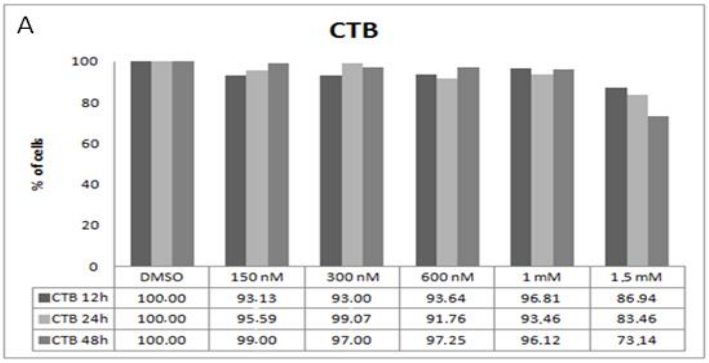


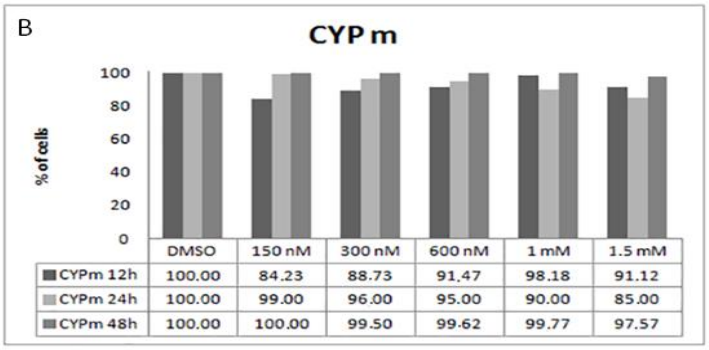
Fig. 21: Immunocytochemistry against CD117, PDGFRα, PDGFRβ and VEGFR2 on canine mammary carcinoma cell lines. (A) CTB cells stained with CD117 antibody (20X magnification), (B) CypM cells stained with PDGFRβ antibody (20X magnification), (C) CypP cells stained with PDGFRα antibody (20X magnification), (D) CTB cells stained with VEGFR2 antibody (20X magnification) (Gattino et al., 2017).

Proliferating assay results

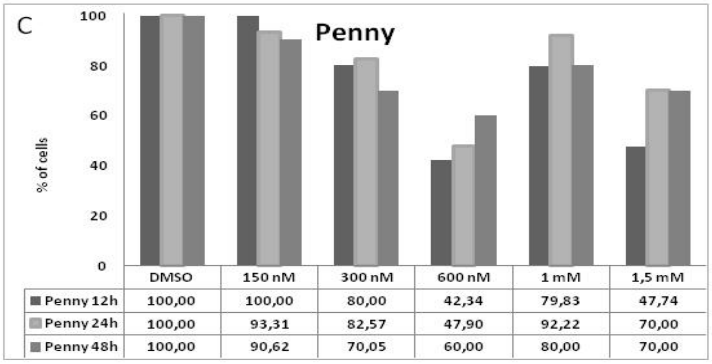
Toceranib phosphate was able to slightly inhibit cell proliferation in CTB cell line at 600 nM (*P*=0.04) after 24 h and at 1.5 mM after 24 and 48 h of treatment (Fig. 22A). In CYPm an inhibition was found only at 1.5 mM after 48 h (Fig. 22B). As shown in Fig. 22C, Penny cell line, which was used as control, responded to toceranib phosphate in a range of 150-600 nM corresponding to IC50 at 12, 24 and 48 h (Liao et al., 2002).



	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



	DMSO	150 nM	300 nM	600 nM	1 mM	1.5 mM
CYPm 12h	100.00	84.23	88.73	91.47	98.18	91.12
CYPm 24h	100.00	99.00	96.00	95.00	90.00	85.00
CYPm 48h	100.00	100.00	99.50	99.62	99.77	97.57



	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

Fig. 22. 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) (Gattino et al., 2017).

Clinical trial Results

In the clinical group of 38 dogs enrolled for the in vivo use of toceranib phosphate (Palladia), 8 were mixed breed, 3 Labrador, 3 Boxer, 3 West Highland White Terrier, 3 Rottweiler, 3 Dachshund, 2 Dobermann and 14 of different breeds. Twenty bitches were entire and 18 neutered. The median body weight was 22 kg and the median age was 10 years. In 27 cases, the diagnosis was histopathological while, in the rest of the sample, confirm was obtained by cytology. In 15 dogs (40.5%), the regional lymph node was found enlarged and aspirated: the cytology confirmed the metastatic disease in all cases.

Histology	Number of cases
Simple carcinoma, III grade	12
Simple carcinoma, II grade	1
Simple carcinoma, unknown grade	12
Carcinosarcoma	1
Complex carcinoma	1
Mixed carcinoma	1

In seven of the 27 histological diagnosis were described neoplastic emboli in the lymphatic vessels.

All dogs were staged before starting the chemotherapy: in 33/38 cases with three-views thoracic radiographs, in 27/38 cases with abdominal ultrasound and in 5 cases with total body CT scan. In 12 cases, pulmonary metastases were reported (one with pleural effusion) and in other 3 cases cutaneous satellite lesions were reported near the primary site.

According the TNM classification 2 cases were reported as stage II, 14 cases as stage III, 10 cases as stage IV and 12 cases as stage V.

The toceranib phosphate (Palladia, Zoetis) median dose administered was 2.98 mg/kg (range from 2.4 to 3.25 mg/kg) every other day. In four cases, the regimen was reduced every Monday, Wednesday and Friday following gastrointestinal toxicity.

In 14 cases (36.8%), adverse events were reported: 69% of cases presented gastrointestinal toxicity (5 cases grade I, 4 cases grade II, 1 case grade III), 23% of cases a hematological toxicity and 8% a musculoskeletal toxicity (1 case grade III).

The mean overall progression free interval was 50.5 days and the median 33 days (Fig. 23).

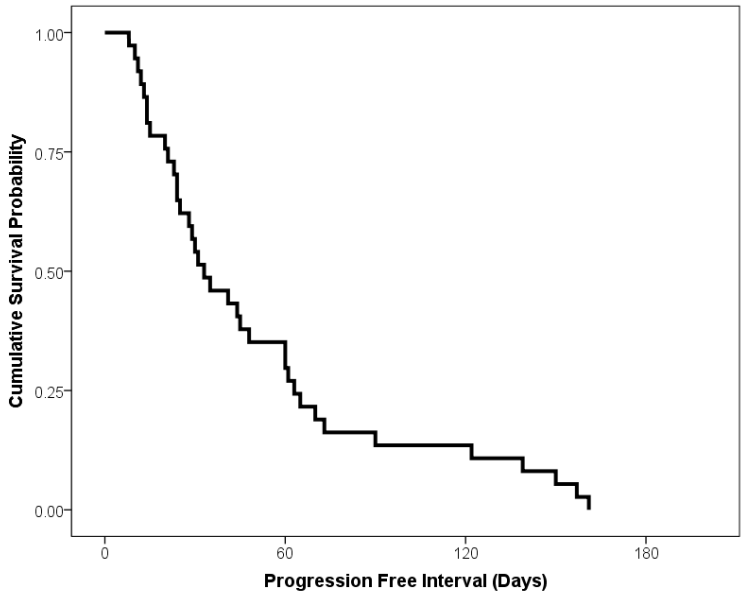


Fig. 23: Overall progression free interval in the 38 dogs treated with toceranib phosphate

The correlation between the different type of clinical response, according the RECIST guideline, and the DFI shown a statistical correlation as reported in Fig. 24 and 25. In Fig.24 were evaluated separately partial response, stable disease and progressive disease, while in Fig. 25 partial response and stable disease were considered in the same group according to London et al., 2003 where a stable disease was considered as a positive result.

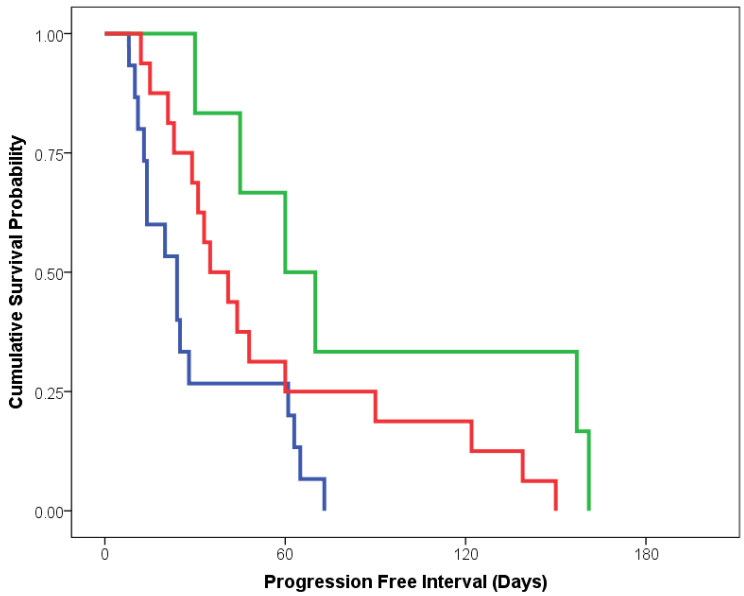


Fig. 24: In this Kaplan Meier, the different Progression Free Interval (PFI) comparing the partial response (green line), the stable disease (red line) and the progressive disease (blue line). The difference is statistically significant.

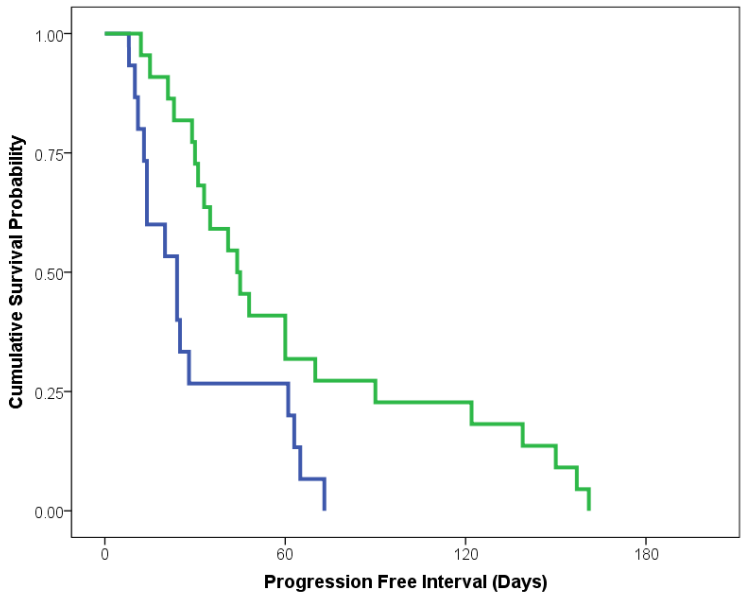


Fig. 2: In this Kaplan Meier the different PFI comparing the partial response and stable disease together (green line) and the progressive disease (blue line). The difference is statistically significant.

The PFI was showed not correlated with dimension of the tumour (Fig. 26), but statistically correlated with the regional lymph node status (P=0.013) (Fig. 27) and with distant metastases (P=0.008) (Fig. 28).

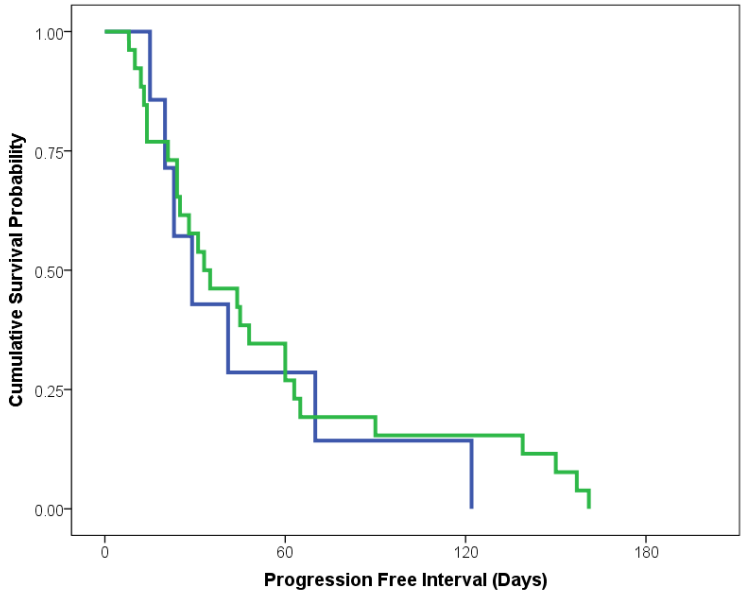


Fig. 26: In the green line tumours < 5 cm and in the blue line tumours > 5 cm.

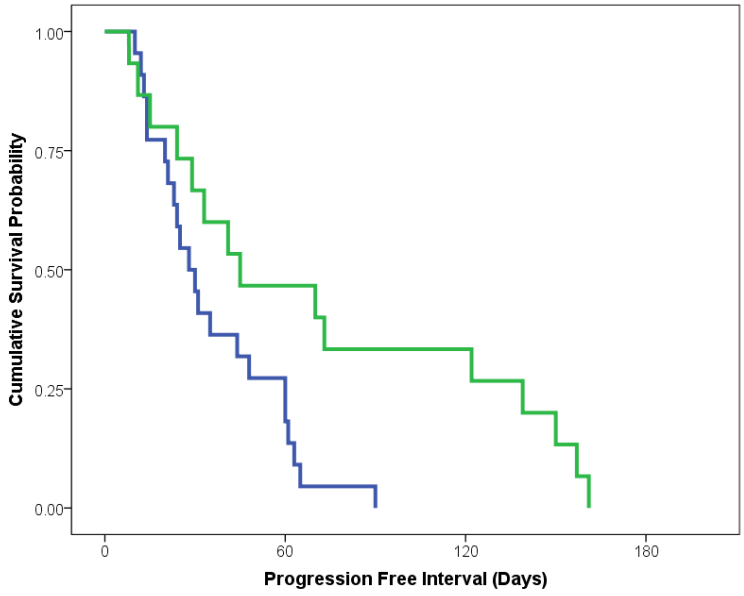


Fig. 27: in the green line not metastatic lymph nodes, in the blue line metastatic lymph nodes.

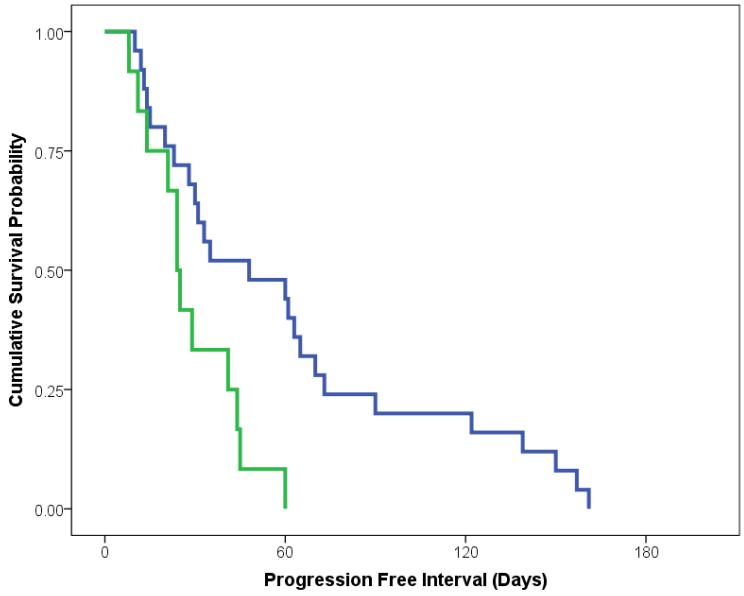


Fig. 28: in the green line metastatic patients, in the blue line not metastatic patients.

Evaluating these data according the study of London et al. (London et al., 2003), seven dogs (19%) had a relevant response more than 10 weeks. Four of these patients had a partial response and three had a stable disease. The histological diagnosis of these dogs were one mixed carcinoma, four simple carcinomas (grade III), one complex carcinoma and one carcinoma of unknown grade. In these seven dogs, the mean PFI was 139 days.

Immunohistochemistry against PDGFR alpha, Beta, VEGFR2 and CD117 was performed only on 11/38 cases because in the other 27 was not possible obtain a surgical sample for the impossibility to perform an anesthesia due different pathological condition, owner's refusal, or because referred from other clinics after the beginning of the protocol. Unfortunately of the seven cases that reported a partial response only on two was performed an IHC evaluation and in both there was a co-expression of PDGFRs and VEGFR-2.

In the group that encountered progressive disease with the Palladia treatment, two of the remaining 9 cases were histologically described as III grade simple carcinomas with neoplastic emboli in lymphatic vessels and both presented a co-expressions of PDGFR alpha and VEGFR-2. The survival data of

these two cases is similar to what described in literature in inflammatory carcinomas with other treatments.

Three cases co-expressed CD117 and VEGFR-2 but the survival data in these three cases was discordant: two showed a progressive disease and only one had a stabilization of the disease for 2 months before the progression. Finally, two cases showed just CD117 expression and one VEGFR-2. One of the two expressing the CD117 positivity shown a partial response for 8 weeks: this case presented a strong membrane positivity compared with the other that was negative at this level.

Case Report Lilly

The following pictures represented the case report of a neutered female Rottweiler dog named Lilly. She presented at first consult for a mammary tumour in the inguinal area, staged as T3 N1 M1, as shown respectively in fig. 29, fig. 30 and 31 A and B. A very inflamed and painful lesion with areas of erythema and ulceration characterized the mammary mass of 15.7 cm. The inguinal lymph node was increased in size (4.28 cm) and metastatic at the cytology. The dog presented also a 3 cm mammary mixed carcinoma, grade II, in the left second mammary gland (pictures not shown), not previously noted by the owner, with a metastatic sternal lymph node (Fig. 31A).

The histology of the inguinal mass was compatible with an anaplastic carcinoma with, neoplastic emboli in the lymphatic vessels (Fig. 32).

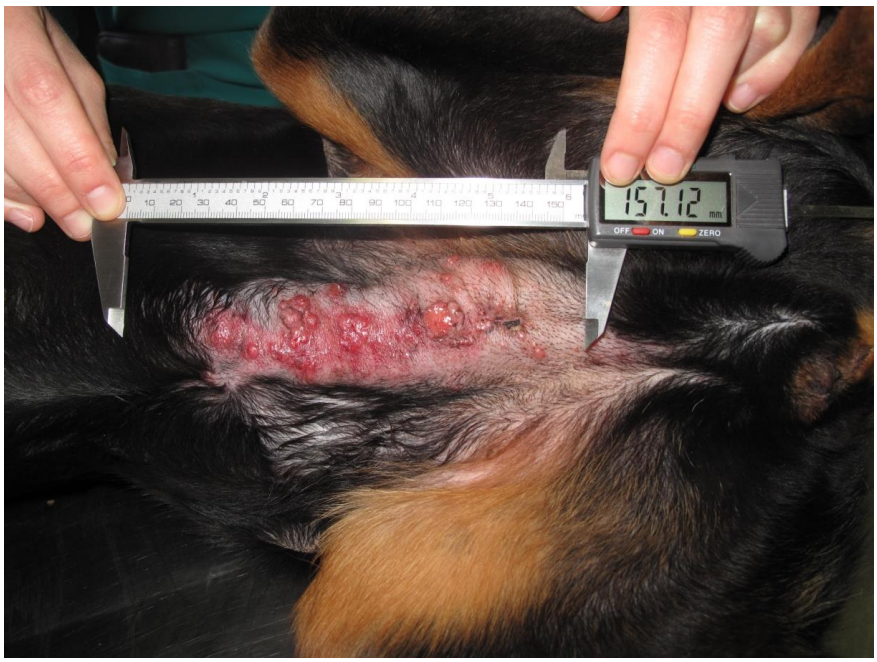


Fig. 29: Lilly case- the inguinal simple carcinoma

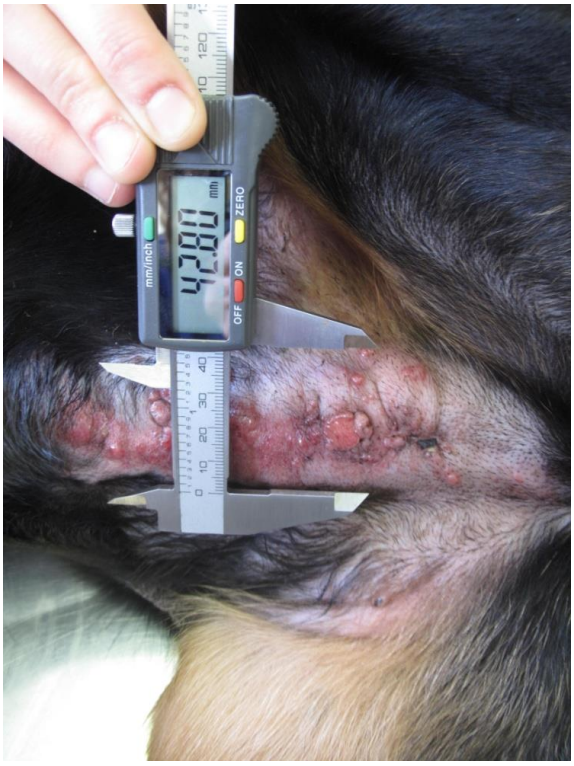


Fig. 30: Lilly case- the inguinal lymph node

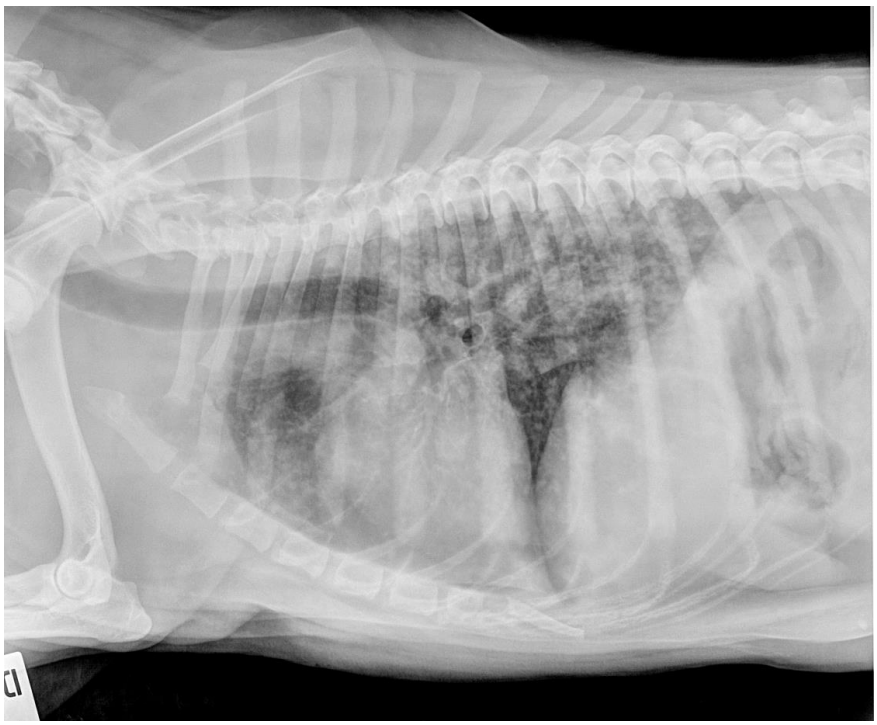


Fig. 31A: Lilly case- megalic sternal lymph node and diffuse pulmonary metastasis

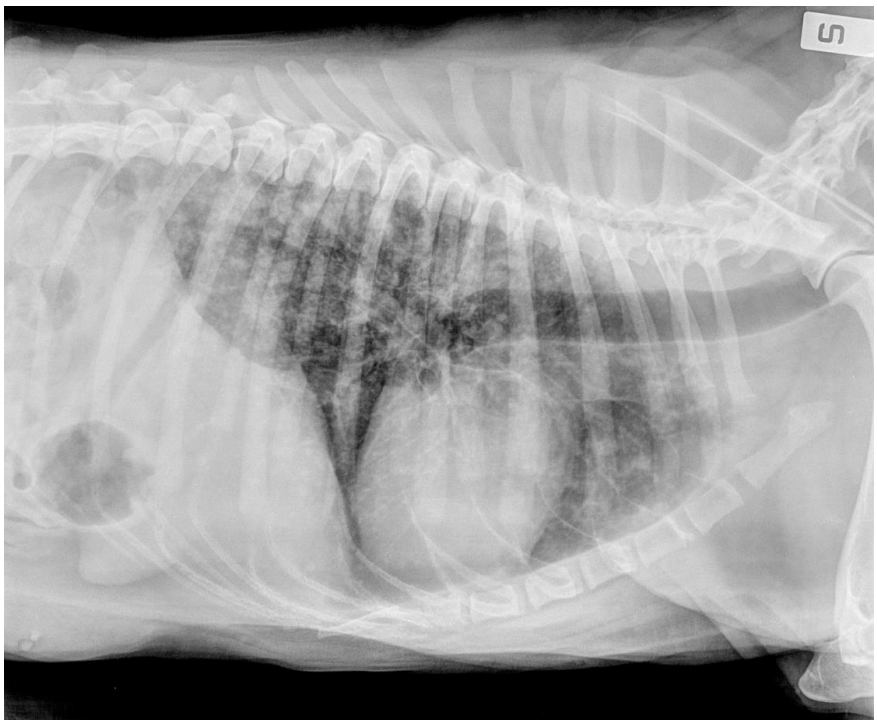


Fig. 31B: Lilly case- diffuse pulmonary metastasis

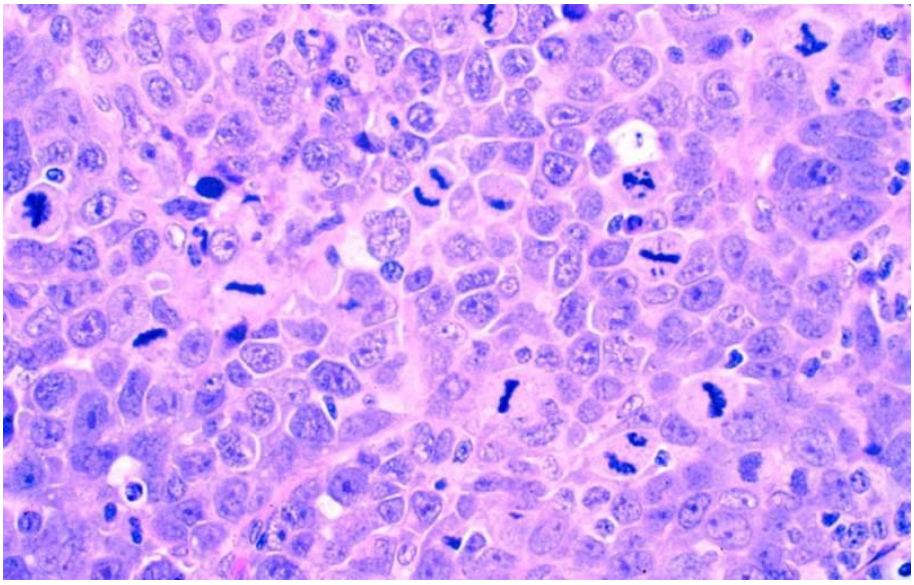


Fig. 32: Lilly case- histology of the anaplastic carcinoma.

Lilly was treated with Palladia at the initial dose of 3.25 mg/kg every other day for two weeks and then reduced a 2.75 mg/kg due the gastrointestinal side effects. The drug was administered with food and gloves. At the first control at 1 month of treatment, the mammary carcinoma was stable in size (Fig. 33) but the pulmonary metastasis were radiographically in partial remission (Fig. 34).

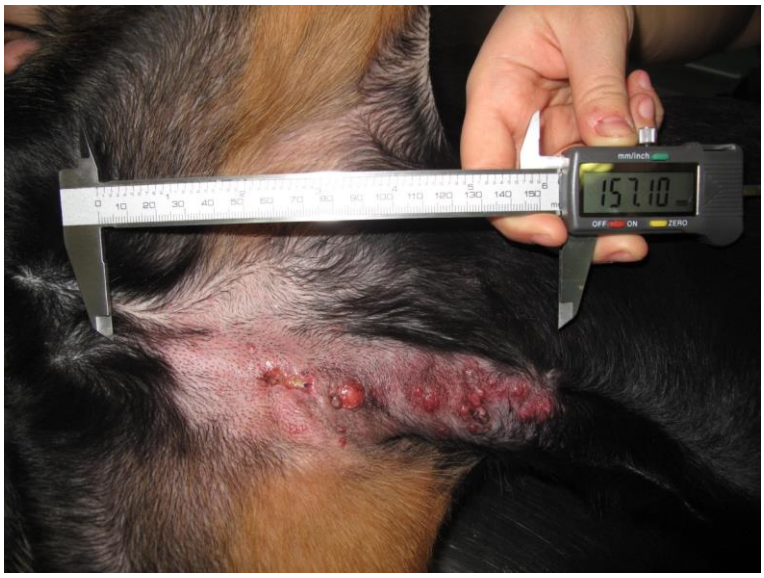


Fig. 33: Lilly case- mammary carcinoma at 1 month of treatment

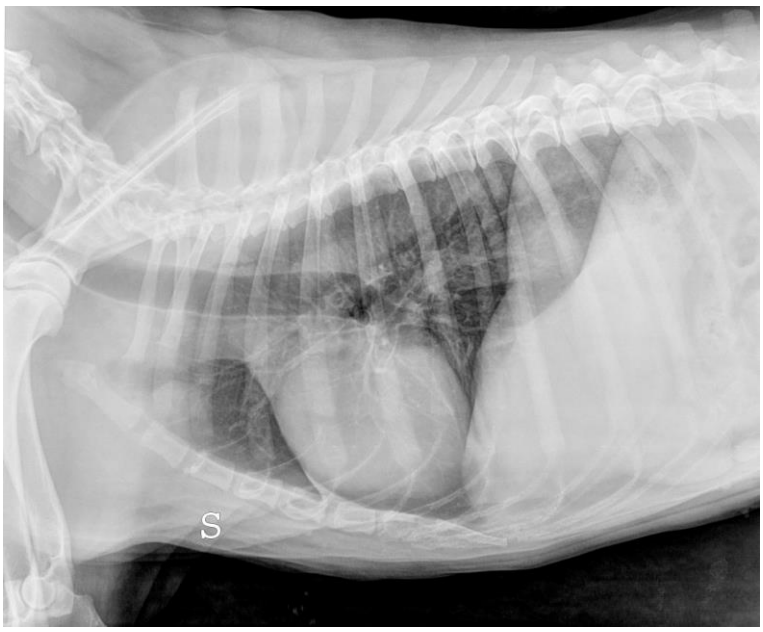


Fig. 34: Lilly case- partial remission of the pulmonary metastasis and stable disease in the sterna lymph node

The clinical control at three months of treatment showed a mild decrease in size of the mammary mass (Fig. 35) but a radiographically complete remission of the distant metastasis and of the sternal lymph node (Fig.36).

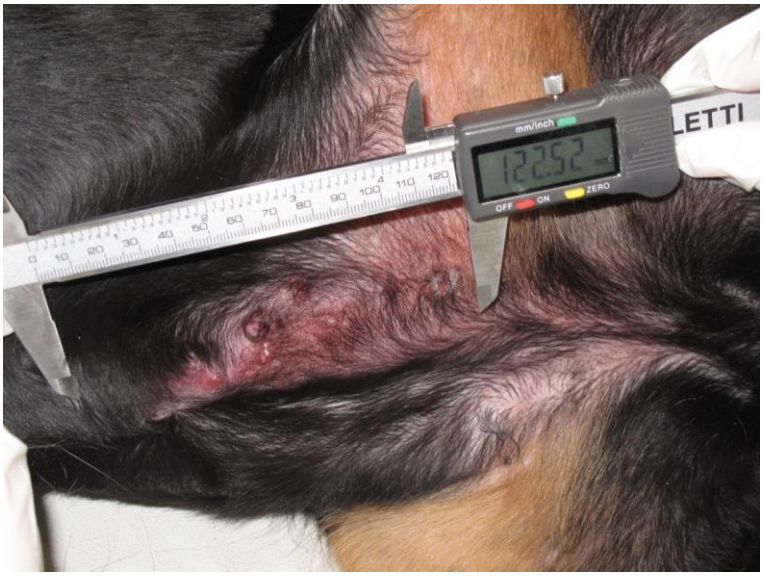


Fig. 35: Lilly case- mammary carcinoma at three month of treatment

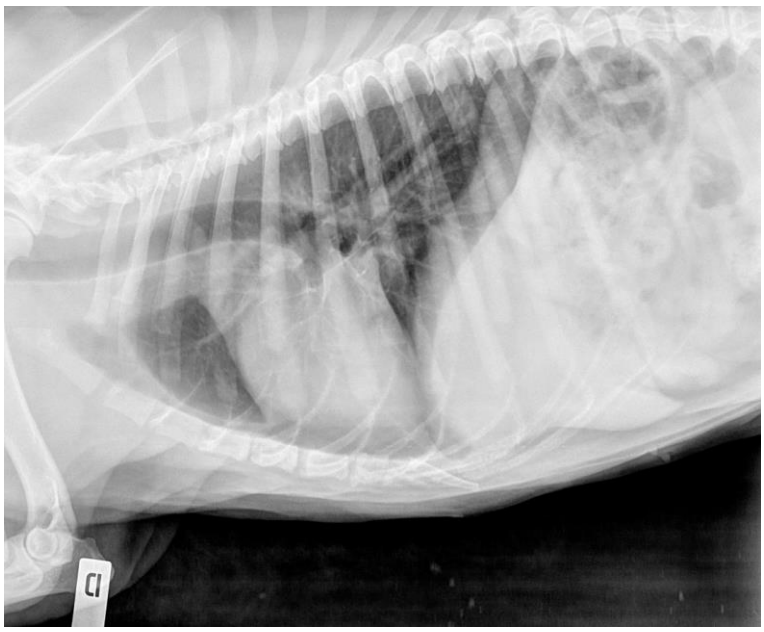


Fig. 36: Lilly case- partial remission of the pulmonary metastasis and of the sterna lymph node

DISCUSSION

Canine mammary tumour is the most frequent diagnosed cancer occurring in entire bitches. The simple carcinoma in particularly, for clinical and pathological findings resembles to human breast cancer and is considered a suitable model in comparative oncology (Liu et al., 2014; Ranieri et al,2013, Vail et al., 2000; Gilbertson, 1983).

Many efforts have been recently made to increase the knowledge of the pathogenesis of CMTs as well as to identify new histological biomarkers for prognosis and specific therapy. Toceranib phosphate (Palladia, Zoetis) is currently used to treat canine mast cell tumours and several other neoplasm in a “off label” use (London et al., 2009; Amagai et al., 2013; Patruno et al., 2014; Gil da Costa, 2015). The efficacy of this drug in CMTs was evaluated only in one study (London et al., 2003). Iin this study, four of the five examined metastatic mixed mammary carcinomas obtained a biological response to therapy. A partial response was see in two dogs with pulmonary metastasis, with a regression of these metastases for 21 and more of 60 weeks; while the other two cases remained on study with stable pulmonary metastases for respectively 27 and 38 weeks. In this study, London et al. reported an objective clinical response in 16 dogs with advanced malignancy and stable disease for more than 10 weeks in 15 additional dogs and that mammary tumours reported the most encouraging data. The author described as not known the mechanisms of action of this medication in CMTs, but reported as the combination in the inhibitions on TKRs had clearly contributed to the observed regression (London et al., 2003).

As shown previously, Palladia generally acts on the phosphorylated form of the target Tyrosine kinase receptors and to obtain the pharmacological benefit, canine patients should need to overexpress/mutate one or more of PDGFRα, PDGFRβ, VEGFR-2 and CD117.

For this reason, 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 et al., 2002; Gujam et al., 2014; Paulsson and Micke, 2014; Pinto et al., 2014; Dekker et al., 2015), epithelial and stromal expressions were evaluated separately.

The results of this study demonstrated the lack of PDGFRα and PDGFRβ expression in more aggressive tumoral istotypes both in neoplastic and in stromal cells, especially for PDGFRβ. In particular, our data demonstrated that PDGFRα and PDGFRβ are less expressed in simple carcinoma respect the complex/mixed carcinomas (P= 0,020 and P=0, 0350 respectively). Furthermore we found that in simple carcinomas, considered in literature more aggressive than mixed/complex carcinomas (Goldschmidt et al., 2011; Pena et al., 2014), PDGFRβ showed a loss of expression in grade III simple carcinoma compared with both I and II grades (P=0,0136).

Instead in the normal/hyperplastic tissue, none of the normal mammary glands expressed PDGFRs, while 54.5% of benign tumours was positive.

This result suggests a possible role of PDGFRs in the early phases of tumorigenesis; nevertheless is possible that the expression of these receptors could be lost with the malignant transformation of the tumour but this data could not be supported due the impossibility of following the receptors status in the tumoural evolution.

Considering specifically simple carcinomas, PDGFRα and β were funded not expressed at the epithelial level in 26/42 and 30/42 (61.9% and 71.4%) of the cases, respectively. These results are partially correlated to human breast cancer, where PDGFR β is expressed exclusively in stromal cells in 35% of cases. In human 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 et al., 2005). Based on 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).

Similar to what is described in the veterinary literature, VEGFR-2 expression was slightly increased in malignant forms (about 55% of cases), but no statistical correlation was found with histological grade (P>0.05). In veterinary literature several studies had investigated VEGFR-2 expression in CMTs especially in correlation to the micro vessel density, VEGF-A expression and clinical follow up, but so far a full agreement between authors have not been reached (Restucci et al., 2004, Al-Dissi 2007, Santos et al., 2014, Diessler et al., 2017). The data in this study are in contrast to those of Restucci and others (2004) that reported a number of positive endothelial and neoplastic cells, higher in malignant tumours compared with the benign neoplasms. This disagreement should be explained by the different methodological approach used to evaluate the positivity of VEGFR-2.In our study we evaluated

separately the expression of this TKR in neoplastic and stromal cells, while Restucci evaluate the total positivity in both. Moreover, our data are similar to those obtained in human, where the immunohistochemically 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, in our study CD117 was expressed in 38.5% of malignant samples without a statistical association with histotype: these data are in contrast with Brunetti and others (2014) that demonstrated an expression of 66.6% of the malignant cases with a statistical association between CD117 labelling pattern and histological type with Ki67 index. This disagreement may be linked to the different number of cases enrolled and to the use of a previous classification (Gilbertson and others 1983), which not evaluated, as histological parameter, the mitosis index which is an essential value in the most recent classification (Goldschmidt et al., 2011). Nevertheless, our data are in agreement with the human literature, where high-grade carcinomas do not express CD117 (Tomasino et al., 2009; Kondi-Pafiti et al., 2010). Therefore, further investigations are necessary to clarify the role of CD117 in this tumour.

The molecular findings of the present study confirmed the antibody specificity and demonstrated that the assessed TKRs are only occasionally expressed in the analyzed cell lines.

Finally, the in vitro assay suggested that the response to drugs is strictly dependent by the presence of specific TKRs on cellular membrane surface. Indeed the *in vitro* treatment by toceranib phosphate did not effects on proliferation activity of CTB and CYPp cell lines. Penny cell line, used as control and overexpressing PDGFRα and PDGFRβ, showed instead a partial response to *in vitro* inhibition at concentration lower than the IC50 value (Maniscalco et al., 2013; Liao AT et al, 2002) demonstrating that the anti-proliferative effect mediated by toceranib phosphate is strictly correlated with the TKRs expression.

The in vivo preliminary results showed in few cases a clinical response (stable disease or partial response) but unfortunately, the majority of the sample had a progressive disease; in these samples, a not significant association with the TKRs expression and clinical response to TOC has been found. Despite this could be important to note as 5 of the 7 dogs that showed a positive response had a very aggressive histotype: one carcinosarcoma and four simple carcinomas (grade III). Comparing this data with the literature, the mean PFI of 139 days could be considered a mild positive result. Unfortunately

in this study was impossible correlate the clinical response in vivo with the receptors status due the small number of histological samples available for molecular studies.

These clinical results are preliminary and not conclusive to suppose a treatment with Palladia in CMTs. For this reason, more efforts need tofocus on the TKRs dysregulation (over expression, mutation or chromosomal translocation) in CMTs in order to evaluate as in humans the use or not of specific TKI that could be useful, well tolerated, and extend the survival time.

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