UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOMEDICINA COMPARATA E ALIMENTAZIONE

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE VETERINARIE

Indirizzo di Sanità Pubblica e Patologia Comparata

XXV Ciclo

INTERACTION BETWEEN TUMOUR AND MICROENVIRONMENT - MOLECULAR MECHANISMS OF CELL MIGRATION IN CANINE TUMOURS

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ABSTRACT

Different steps forward have been made in the recent years to identify the molecular determinants in carcinogenesis and the evidence of a multistep process where cancer cells accumulate multiple and consecutive genetic alterations has been formulated. Recently, tumour progression has been recognized as the product of a complex crosstalk between tumour cells and their surrounding and supporting tissue, named tumour stroma.

This stroma is known to influence the growth of cancer and it is composed by several types of cells, including endothelial cells of blood and lymphatic circulation, stromal fibroblasts and a variety of bone marrow-derived cells, such as macrophages, mast cells, neutrophils, lymphocytes and mesenchymal stem cells. The supportive microenvironment is generate and modulated by cancer cells through the production and activation of stroma growth factors including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). Concomitant with altered growth-factor expressions, induced by their autocrine and paracrine effect on the tumour and stromal cells, cancer cells are able to produce proteolytic enzymes, such as Matrix metalloproteinases (MMPs), which operate the remodelling of extracellular matrix (ECM) and basement membrane, thus activating cell-surface and ECM-bound growth factors. All these processes are described to contribute to the extensive crosstalk between the microenvironment and the cancer cells.

Therefore, the microenvironment is implicated in the regulation of cell growth, determining angiogenesis, tumour invasion and metastasis, and impacting the outcome. Even if stromal cells are not malignant, their role in supporting cancer growth is vital to the survival of the tumour. For this purpose, cells of microenvironment have become an attractive target for therapeutic agents.

The present project has been divided in different tasks to identify the molecular mechanisms implicated in cell migration, angiogenesis and tumour growth led by stroma cells and their crosstalk with cancer cells in different neoplasia in dog. Canine mammary tumour, cutaneous mast cell tumour, lymphoid leukaemia and lymphoma were selected for the study and gene expression profiling and proteomic analysis of different growth factors (VEGF-TGF-β-PDGF) and MMPs were analyzed in association with their possible prognostic and predictive role and crosstalk.

Several important results have obtained highlighting the background of the tumour progression and the role of microenvironment in veterinary oncology. Selected results are shown below:

- MMP-2, MT1-MMP, MMP-9 were significantly involved in canine mammary tumour and a significant role of the stromal compartment was described;
- MMP-9 and VEGF-A were associated with the histological tumour grade in cutaneous mast cell tumour;
- MMP-9, MT1-MMP, TIMP-1 and VEGF were correlated in T-cell lymphoma and in dogs with higher stage;
- A potential role of MT1-MMP and TIMP-2 in the pathogenesis of canine acute lymphoblastic leukaemia has been discovered;
- In chronic lymphocytic leukaemia, residual normal leukocytes have shown a significative influence in the expression of MMP-9, MT1-MMP, VEGF and TIMPs;
- Lymphoma and leukaemia in vitro model exhibited a significative discrepancy that enhanced the importance of microenvironment in vivo;
- PDGF-B mRNA expression was identified in canine T-cell lymphoma and cutaneous lymphomas. A functional autocrine and/or paracrine loop of growth stimulation was proposed due to the co-expression of PDGFs and PDGFRs at different time point during disease.

Therefore, the obtained results may significantly improve the understanding of cancerogenesis of the most frequent tumours in dogs. The summarized data here show a primary role for the microenvironment during carcinogenesis. Development of novel cancer therapies that target the process of metastasis formation, tumour growth and differentiation, by interfering with the ability of cancer cells to transmigrate into blood and lymph vessels and to invade the connective tissue, is widely expected in veterinary oncology. Further data are necessary to indicate that the use of chemopreventive agents to control the function and behaviour of cells in the microenvironment might be an important approach to the overall control of cancer.

RIASSUNTO

Negli ultimi anni nell'ambito dell'oncologia, diversi studi hanno identificato diverse molecole target implicate nella cancerogenesi e sono stati evidenziati numerosi processi attraverso cui le cellule tumorali sono in grado di accumulare alterazioni genetiche. Recentemente, la progressione del tumore è stata riconosciuta come il prodotto di un complesso crosstalk tra le cellule tumorali e il tessuto circostante, chiamato stroma tumorale.

Questo stroma è noto per influenzare la crescita del tumore ed è composto da diverse tipologie cellulari, che comprendono cellule endoteliali della circolazione sanguigna e linfatica, fibroblasti stromali ed una varietà di cellule derivate dal midollo osseo, come macrofagi, mastociti, neutrofili, linfociti e cellule staminali mesenchimali. Ulteriormente, il microambiente di supporto è generato e modulato da cellule tumorali attraverso la produzione e attivazione di fattori di crescita prodotti dallo stroma stesso, come Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF) e Transforming Growth Factor-β (TGF). Concomitante all'alterata espressione di questi fattori e per il loro effetto autocrino e paracrino sulle cellule tumorali e su quelle stromali, le cellule neoplastiche iniziano a produrre enzimi proteolitici, come metalloproteasi di matrice (Matrix metalloproteinases - MMPs). Le MMPs operano il rimodellamento della matrice extra cellulare e della membrana basale, attivando così fattori di crescita legati alla superficie cellulare e alla matrice stessa. Tutti questi processi contribuiscono all'esteso crosstalk tra il microambiente e le cellule tumorali.

Il microambiente quindi è implicato nella regolazione della crescita cellulare, determinando neoangiogenesi, invasione, metastasi tumorali e influenzando il risultato della terapia. Anche se le cellule stromali non sono considerabili fenotipicamente maligne, il loro ruolo nel sostenere la crescita della neoplasia è essenziale per la sopravvivenza del tumore. Con questo presupposto, le cellule del microambiente sono diventate un bersaglio attrattivo per diversi agenti terapeutici.

Il progetto di ricerca è stato suddiviso in diverse fasi per identificare i meccanismi molecolari implicati nella migrazione cellulare, nell'angiogenesi e nella crescita neoplastica, da parte di cellule stromali e dal loro crosstalk con le cellule tumorali, in diverse neoplasie del cane. Per lo studio sono state selezionate le tipologie tumorali più frequenti nel cane: tumore mammario, mastocitoma cutaneo, leucemie linfoidi e linfoma, analizzando i profili di espressione genica e

proteica di diversi fattori di crescita (VEGF-TGF-β-PDGF) e delle MMPs, in associazione al loro crosstalk e ad un loro eventuale ruolo prognostico.

Sono stati ottenuti importanti risultati evidenziando lo scenario della progressione tumorale e il ruolo del microambiente in oncologia veterinaria. E' stato dimostrato che:

- MMP-2, MT1-MMP, MMP-9 sono significativamente coinvolte nel tumore mammario ed è stato descritto un loro ruolo rilevante del compartimento stromale;
- MMP-9 e VEGF-A sono associati al grado istologico nei mastocitomi cutanei;
- MMP-9, MT1-MMP, TIMP-1 e VEGF sono correlate nel linfoma T e nei cani con linfoma con stadio clinico più alto;
- MT1-MMP e TIMP-2 hanno un ruolo nella patogenesi nelle leucemie linfoblastiche acute;
- Nella leucemia linfocitica cronica, i leucociti residui normali mostrano un'influenza significativa nell'espressione di MMP-9, MT1-MMP, VEGF e dei TIMPs;
- Il linfoma e la leucemia nel modello in vitro mostrano una considerevole discrepanza per alcune MMPs e VEGF che avvalora l'importanza del microambiente in vivo;
- L'espressione genica del PDGF-B è significativa nei linfomi T e nei linfomi cutanei. E' stato inoltre proposto un loop funzionale autocrino e/o paracrino di stimolazione della crescita della neoplasia, dovuto alla co-espressione dei PDGFs e dei recettori in diversi tempi durante la malattia.

I risultati ottenuti potrebbero migliorare significativamente la comprensione della cancerogenesi nei tumori più frequenti nel cane. I dati qui sintetizzati mostrano un ruolo primario del microambiente durante la carcinogenesi. Lo sviluppo di nuove terapie antitumorali che colpiscano il processo di formazione di metastasi, la crescita e la differenziazione della neoplasia, interferendo con la capacità delle cellule tumorali di trasmigrare nel sangue e nei vasi linfatici e di invadere il tessuto connettivo, sarà ampiamente perseguito in oncologia veterinaria nel prossimo futuro. Sono però necessari ulteriori studi per indicare se l'uso di agenti chemio-preventivi per controllare la funzione ed il comportamento delle cellule nel microambiente possa essere un importante approccio al controllo complessivo del cancro.

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ABBREVIATIONS

ALL = acute lymphoblastic leukaemia

a.u. = arbitrary unit

CD = cluster of differentiation

CGI-119 = canine transmembrane BAX inhibitor motif containing 4

CL = cutaneous lymphoma

CLL = chronic lymphocytic leukaemia

CP = crossing point

DLBCL =diffuse large B-cell lymphoma

ECM = extra-cellular matrix

ELISA = enzyme-linked immunosorbent assay

FNA = fine-needle aspirate

GOLGA1 = golgin a 1

GZ = gelatine zymography

HG = high grade

HIF = hypoxia-inducible factor

hNHL = human non-Hodgkin's lymphoma

ICC = immunocytochemistry / chemical

IHC = immunohistochemistry /chemical

LG = low grade

LL = lymphoblastic lymphoma

MCT = mast cell tumour

MMP = matrix metalloproteinase

MT-MMP = membrane-type matrix metalloproteinase

MZL = marginal zone lymphoma

PDGF = platelet-derived growth factor

PTCL = peripheral T-cell lymphoma

qRT-PCR = quantitative real time polymerase chain reaction

RECK = reversion- inducing cysteine-rich protein with Kazal motifs

TGF-β = transforming growth factor beta

TIMP = tissue inhibitor of metalloproteinase

UPL = universal probe library

VEGF = vascular endothelial growth factor

1. BACKGROUND

Cancer is one of the causes of disease and mortality worldwide. The past two decades of biomedical research have collected an enormous amount of information on the molecular events that take place during carcinogenesis and tumour progression. The molecular mechanisms of the complex interplay between the tumour cells and the tumour microenvironment have been identified playing a pivotal role in this process (Gialeli et al. 2010). A variety of stromal cells in the surrounding environment are recruited to tumours, and these not only increase the growth of the primary cancer but also facilitate its metastatic dissemination to distant organs (Joyce and Pollard, 2009) (Fig. 1). Through different mechanisms, the neoplastic cells are able to disseminate by lymphatic and blood circulation, and then create metastatic growth in distant organs. To spread within the tissues, tumour cells use migration mechanisms that are similar, if not identical, to those that occur during physiological processes such as embryonic morphogenesis, would healing and immune-cell recruitment (Friedl and Bröcker, 2000).

Moreover, the cell body is modified in shape and stiffness during the migration to interact with the surrounding tissue structures. *In vitro* and *in vivo* observations have shown that tumour cells infiltrate neighbouring tissues in diverse patterns. They can disseminate in an “individual cell migration”, or expand in solid cell strands, sheets, files or clusters (called “collective migration”). Leukaemias, lymphomas and solid stromal tumours, such as sarcomas, usually disseminate via single cells; whereas epithelial tumours follow collective migration mechanisms. Such differences in cellular patterning reflect variations in the molecular repertoire used by a cancer cell to migrate (Friedl and Wolf, 2003).

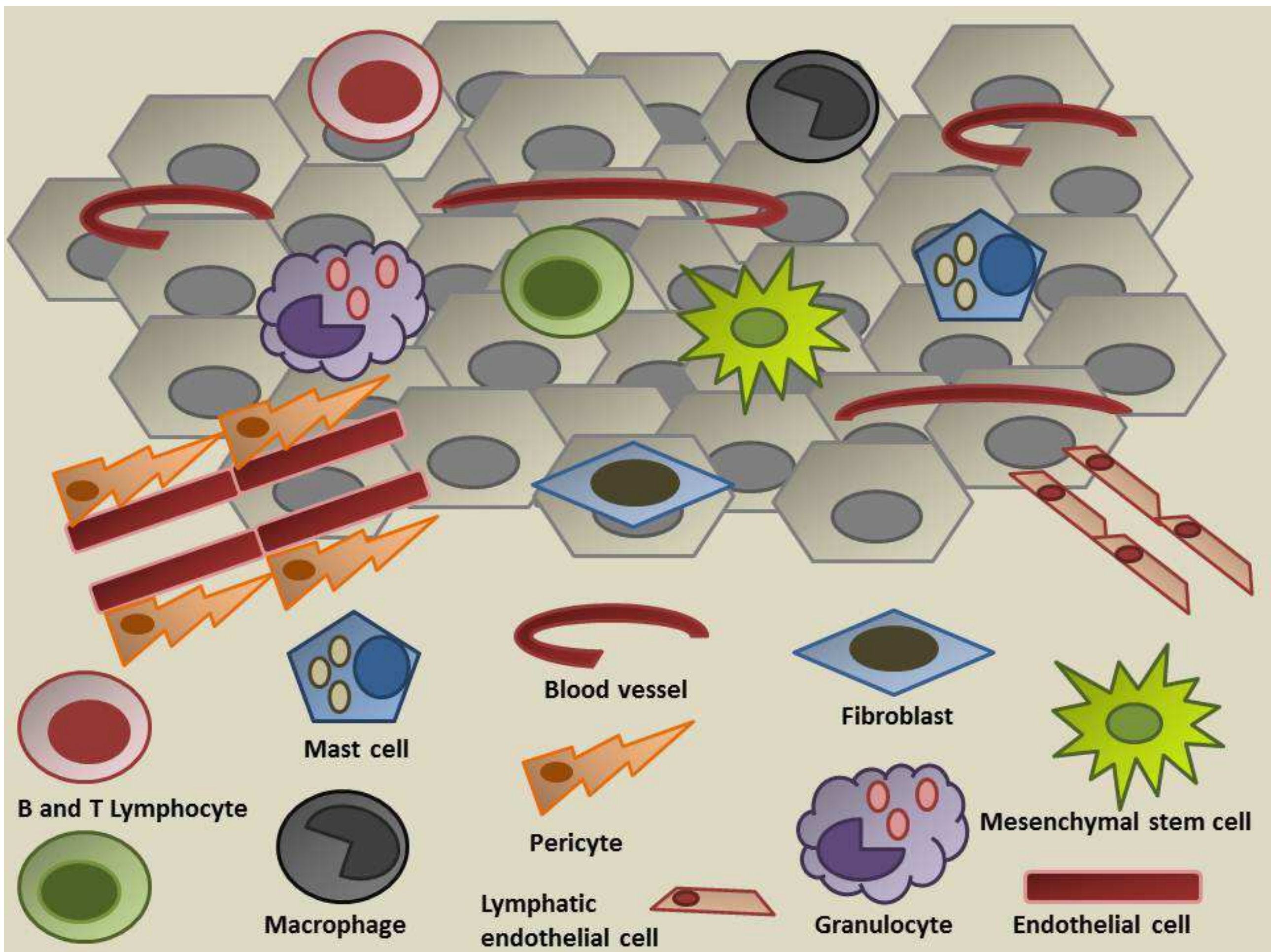

Fig. 1. Primary tumour and microenvironment. Cancer cells in primary tumours have a strict interaction with their supportive microenvironment. The environment is composed by some structural cells including endothelial cells of blood and lymphatic circulation, stromal fibroblasts and a variety of bone marrow-derived cells, including macrophages, mast cells, neutrophils, lymphocytes and mesenchymal stem cells.

MECHANISMS OF TUMOUR-CELLS INVASION AND MIGRATION

In vitro studies have led to the original observations that individual tumour cells are motile. These cells usually originate from the interstitial stroma or bone marrow. Alternatively, cells that arise from a multicellular compartment, such as epithelium, lose their cell contacts, detach and migrate as individual cells through the neighbouring connective tissue. Based on cell type, integrin involvement, cytoskeletal structure and protease production, single-cell migration can occur in different morphological variants (Thiery, 2002). These variants include:

- *Mesenchymal migration.* Mesenchymal movement is predominantly found in cells from connective-tissue tumours, such as fibrosarcoma, glioma and in epithelial cancers, the latter characterized by a progressive dedifferentiation. The cells following this type of cellular patterning have a fibroblast-like spindle-shaped morphology that is dependent on integrin-mediated adhesion dynamics and the presence of high traction forces on both cell poles.
- *Amoeboid migration.* The characteristics of amoeboid movement have been established through studies of the single-cell amoeba, *Dictyostelium discoideum*. *Dictyostelium* is an elliptoid cell that translocates with morphological expansion and contraction; it has an extraordinary deformability and relatively low-affinity substrate binding that is integrin independent. In higher eukaryotes, signs of amoeboid movement are shown in leukocytes and some tumour cells. In lymphocytes and neutrophils, integrin-mediated adhesion is useful for cell migration within connective tissue, both *in vitro* and *in vivo*. T lymphocytes use protease-independent physical mechanisms to overcome matrix barriers. Furthermore an amoeboid dissemination characterizes the early detachment and metastatic spread from a small primary tumour, such as lymphomas and small-cell lung carcinomas.
- *Chain migration.* Chain migration occurs in non-neoplastic neural crest cells, myoblasts, breast carcinoma and melanomas. These cells stream one after another in a strand-like fashion (Friedl and Wolf, 2003).

Cancer cells generate a supportive microenvironment by producing stroma-modulating growth factors including the vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). Similar to the processes of wound healing, these factors disrupt normal tissue homeostasis and act in a paracrine manner to induce stromal reactions such as angiogenesis and inflammatory response. Moreover, they can activate

surrounding stromal cells, such as fibroblasts, smooth-muscle cells and adipocytes, leading to the secretion of additional growth factors and proteases (Mueller and Fusenig, 2004). Concomitant with altered growth-factor expressions, often induced by their autocrine effect on the tumour cells, cancer cells initiate to produce proteolytic enzymes, such as Matrix metalloproteinases (MMPs) (Stetler-Stevenson and Yu, 2001). MMPs operate the remodelling of ECM and basement membrane, exposing cryptic protein domains and generating specific new molecule fragments that can have promigratory as well as pro- and anti-angiogenic functions. Moreover these proteases activate cell-surface and ECM-bound growth factors, during ECM degradation, that contribute to the extensive crosstalk between the microenvironment and the cancer cells (Mueller and Fusenig, 2004) (Fig. 2).

Therefore progression of tumour is a highly complex process in which several molecular events are required for tumour cells to achieve independent growth. Another such event is the enhancement of angiogenesis. Angiogenesis is the development of new blood vessels from existing ones, as opposed to vasculogenesis, which refers to the de novo formation of blood vessels (Levitovic et al., 2006). It is necessary for persistent tumour growth, because the sprouting capillaries are conduits for gas exchange and nutrient supply. The process of angiogenesis is governed by a strict molecular interaction, and its modulation is dependent on angiogenic factors, cytokines, integrins and ECM components that surround the involved vessels (Yoon et al., 2003). These interactions concern cell-to-cell and cell-to-ECM. The induction and rate of angiogenesis depend on the balance of two functionally opposing groups of cytokines called angiogenic and angiostatic (or antiangiogenic) factors (“angiogenesis switch model’’) (Levitovic et al., 2006).

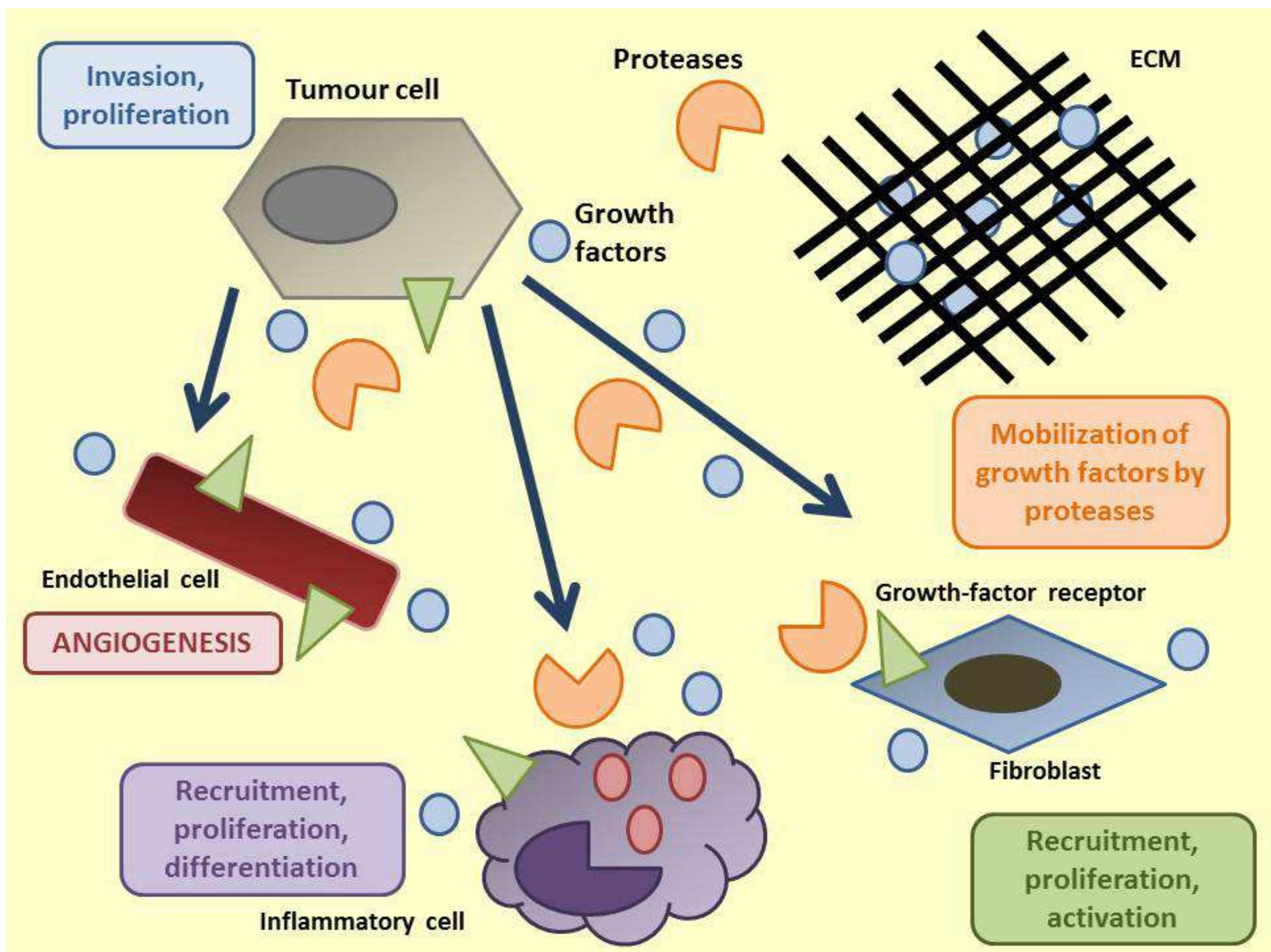

Fig. 2. Crosstalk between tumour cells and their reactive stromal cells. Tumour cells create a supportive microenvironment by secreting growth factors, proteases and their inhibitors, whose imbalance leads to the degradation of ECM, resulting in the release of growth factors, bound to the matrix, and of ECM molecular fragments. The secretion can act both in autocrine and paracrine manners to the stroma, inducing angiogenesis, invasion and proliferation of tumour cells. In addition to recruitment, proliferation and activation of stromal inflammatory cells and fibroblasts are activated, secreting further growth factors and proteases. This cycle permit to amplify these signals in the cascade that results in the establishment of an activated stroma that promotes malignant tumour growth.

1.1. MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are extracellular matrix remodelling proteinases, which belong to a zinc-dependent family of endopeptidases. The MMP family consists of about 24 members that are characterized in humans, rodents, and amphibians. Initially, MMPs are classified according to their modular domain structure and ECM specificity. In particular, the gelatinases are a subgroup within the MMP family and include MMP-2 (gelatinase A or Mr 72,000 type IV collagenase) and MMP-9 (gelatinase B or Mr 92,000 type IV collagenase). They have been described in the tumour invasion and progression in different human tumours (Egeblad and Werb, 2002). In addition, MMPs cleave a lot of substrates, including an array of other proteinases, proteinase inhibitors, chemotactic molecules, latent growth factors, growth factor binding proteins, cell surface receptors, and cell-cell and cell-matrix adhesion molecules. Thereby they regulate cell behaviour in several ways. Therefore, MMPs influence diverse physiologic and pathologic processes. In normal physiology, MMPs are involved in embryonic development, wound repair, ovulation, bone remodelling, macrophage and neutrophil function. In pathologic conditions, such as rheumatoid arthritis, periodontal disease, osteoarthritis, gastric ulcer, arteriosclerosis and then tumour invasion, metastasis and angiogenesis in cancer, MMPs operate an excessive and considerable degradation of ECM (Yoon et al., 2003) (Fig. 3).

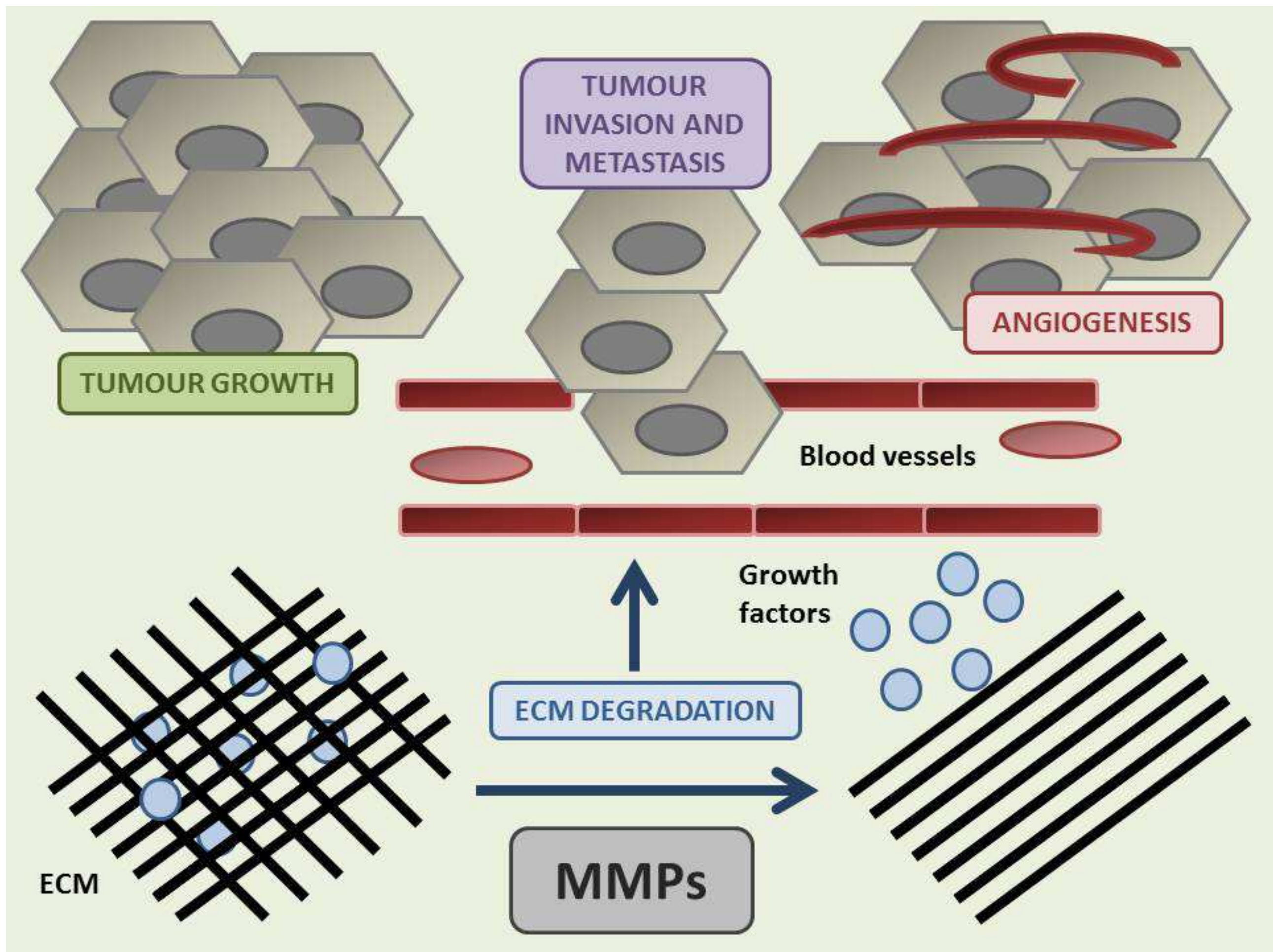

Fig. 3. Roles of MMPs in cancer progression. MMPs can modulate several phases of cancer progression, including tumour growth and survival, tumour invasion and metastasis at the distant sites and angiogenic switch. MMPs exert their proteolytic activity degrading the physical barriers and liberating growth factors, thereby facilitating all these phases.

1.1.1. REGULATION

In the neoplastic process, the MMPs are regulated in a paracrine manner by growth factors and cytokines secreted both by tumour infiltrating inflammatory cells and tumour or stromal cells. This regulation is governed by the continuous crosstalk between tumour cells, stromal cells, and inflammatory cells during the invasion process. Most MMPs are secreted as latent precursors (zymogens) that are proteolytically activated in the extracellular space, with the exception of MMP-11 and Membrane type-1 Matrix Metalloproteinases (MT1-MMP or MMP-14), which are activated prior to secretion by Golgi-associated, furin-like proteases (Westermarck and Kahary, 1999). Endogenous inhibitors tightly control MMP activity. The main inhibitor of MMPs is α2-macroglobulin, an abundant plasma protein. In a similar way to α2-macroglobulin, thrombospondin-2 forms a complex with MMP-2 and facilitates endocytosis and clearance. By contrast, thrombospondin-1 binds to pro-MMP-2 and -9 and directly inhibits their activation.

The most-studied endogenous MMP inhibitors are Tissue Inhibitors of MMPs (TIMPs) -1, -2, -3 and -4, which reversibly inhibit MMPs in a 1:1 stoichiometric fashion. They differ in tissue-specific expression and ability to inhibit various MMPs (Egeblad and Werb, 2002). Although TIMPs inactivate MMPs *in vitro*, the role of TIMPs in the regulation of MMP activity and cancer progression *in vivo* is unclear. Initial studies have shown decreased expression of TIMPs at the site of tumour invasion, but a positive correlation between TIMPs and MMPs expression and poor prognosis in malignant tumours has recently been shown. The contradictory roles of TIMPs may result from the bimodal function of TIMPs as inhibitors, but also as key players in the activation of MMPs. To exert their inhibiting or activating functions, TIMP-1 and TIMP-2 preferentially bind to MMP-9 or MMP-2, respectively (Westermarck and Kahary, 1999).

In addition to TIMPs, the recently characterized RECK (reversion inducing cysteine-rich protein with Kazal motifs) protein, a novel plasma-membrane-anchored MMP inhibitor, has been described to regulate MMPs activity. RECK controls three members of the MMP family (MMP-2, MMP-9 and MT1-MMP) by various mechanisms, inhibiting the secretion or directly the catalytic activity (Takahashi et al., 1998).

MMP gene expression is primarily regulated at the transcriptional level or through the modulation of mRNA stability in response to growth factors and cytokines. In fact, the promoter regions of inducible MMP genes (MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, MMP-13) show

remarkable conservation of regulatory elements, and their expression is induced by growth factors, cytokines, and other environmental factors such as contact to ECM (Yoon et al., 2003).

The pro-MMP-2 can be activated by several mechanisms that are dependent on stimulators and cell types. MMP-2 is activated at the cell surface through a unique multistep pathway that involves MT1-MMP and TIMP-2: TIMP-2 binds MMP-14 at its amino terminus and pro-MMP-2 at its carboxyl terminus, which allows an adjacent, non-inhibited MMP-14 to cleave the bound pro-MMP-2 (Egeblad and Werb, 2002).

1.1.2. TUMOUR CELL INVASION, MIGRATION AND PROGRESSION

High expression levels of certain MMPs were correlated to the tumour invasion capacity *in vivo*. Because of their ECM-degrading activity, MMPs were initially thought to facilitate tumour cell metastasis by destroying the basement membrane and other components of ECM. MMPs also participate in cell-surface proteolysis, leading to the release of different growth regulators and the extracellular domains of proteins from the cell surface (Yoon et al., 2003). Moreover, MMPs act on the non-matrix substrates (e. g., chemokines, growth factors, growth factor receptors, adhesion molecules, and apoptotic mediators) that operate the rapid and critical cellular responses, which are required for tumour growth and progression (Westermarck and Kahary, 1999).

Furthermore, during invasion, the localization of MMPs to specialized cell surface structures is essential for their ability to promote invasion. Specialized cell surface structures utilize transmembrane proteinases, including MT1-MMP, as well as secreted and activated MMPs at the site, such as MMP-2 and -9, to degrade a variety of ECM macromolecules and facilitate cell invasion (Gialeli et al,. 2011).

1.1.3. IMMUNOLOGIC ESCAPE

Once the tumour cell has entered the circulation, the immune system is responsible for the elimination of the circulating tumour cells. In order to be successful in the establishment of metastases, a tumour cell is able to adopt mechanisms of immune escape. MMPs help this process stimulating cancer cell-platelet interactions and inhibition of functions, as well as the proliferation of immune responsible cells, such as the T cell and natural killer cells (Westermarck and Kahary, 1999). Moreover MMPs shed interleukin-2 receptor-a by the cell surface of T-lymphocytes, thereby suppressing their proliferation; then TGF-b, a significant suppressor of T-lymphocyte reaction against cancer cells, is released as a result of MMP activity (Gialeli et al., 2011).

1.1.4. ANGIOGENESIS

MMPs are essential regulators during various phases of the angiogenic process, from the deposition and breakdown of the basement membrane of vascular structures, to the endothelial cell proliferation and migration (Westermarck and Kahary, 1999). The principal MMPs that participate in tumour angiogenesis are mainly MMP-2, -9 and MT1-MMP (Gialeli et al., 2011). In particular, besides its proteolytic degradation, MMP-9 can release angiogenic factors bound to ECM. While tumour cells may produce latent pro-MMPs, in most tumours, the stromal cells and vascular endothelial cells are the major source of pro-MMPs that are activated by distinct MMPs or other proteases (Moehler et al., 2003).

1.1.5. TARGETED THERAPY IN HUMAN

On the basis of the pivotal roles that MMPs play in several steps of cancer progression, the pharmaceutical industry has invested considerable effort over the past 20 years aiming to develop safe and effective agents targeting MMPs. In this regard, multiple Matrix Metalloproteinases Inhibitors (MMPIs) have been developed, in the attempt to control the synthesis, secretion, activation and enzymatic activity of MMPs. Several generations of synthetic MMPIs were tested in phase III clinical trials in humans, including peptidomimetics (like Marimastat in breast cancer), nonpeptidomimetics inhibitors and tetracycline derivatives (like Metastat, also called COL-3, in Kaposi's sarcoma), which target MMPs in the extracellular space (Gialeli et al., 2011). In addition, various natural compounds, like Neovastat in breast and colorectal tumour, have been identified as inhibiting MMPs. Other strategies of MMP inhibition in development involve antisense and small interfering RNA (siRNA) technology. Antisense strategies are directed selectively against the mRNA of a specific MMP, resulting in decrease of RNA translation and downregulation of MMP synthesis (Mannello, 2006). However the road to clinical use of MMPIs has not been straightforward. Several reasons might explain the unfavourable clinical outcomes in some of the MMPI clinical trials in various cancer types. Firstly, adverse effects, including musculoskeletal syndrome, have limited the maximum-tolerated dose of the early generation of MMPIs, thereby limiting drug efficacy. Secondly, patients recruited in the trials are often at the most advanced and metastatic stage of cancer. In addition, the nonspecific nature of the inhibitors has a negative impact in their therapeutic efficacy, due to the wide range of MMPs and physiological events affected (Roy et al., 2009). Considering all of the above, one of the major challenges for the future is the development of inhibitors or monoclonal antibodies that bind to the active site of the

enzyme and are specific for certain MMPs, showing little or no cross-reaction with other MMPs (Gialeli et al., 2011).

1.1.6. MMPs IN DOG

Although evidence for the role of MMPs in tumours of domestic animals is sparse, the central role of MMP-2 and MMP-9 has been documented in many pathologies. The sequence homology between other species and human MMPs is high; therefore, the use of human standards to estimate the location and presence of canine MMPs appears reasonable. There is a 96% to 98% sequence homology among rat, mouse, and human MMP-2, whereas there is a 75% to 85% sequence homology among rat, mouse, rabbit, human, and bovine MMP-9. An experimental work to sequence canine MMP-2 has revealed high sequence homology with human, rat, and mouse sequences (Leibman et al., 2000). Furthermore Coughlan et al. (2000) have shown the N-terminal amino acid sequence of canine MMP-2 to have 87% homology with the human sequence. Increased MMP-2 and MMP-9 levels occur in the synovial fluid in canine rheumatoid arthritis (Coughlan et al., 1998) and increased MMP-2 levels in canine osteoarthritis (Coughlan et al., 1995), whereas increased pro–MMP-9 levels occur in the myocardium in canine dilated cardiomyopathy (Gilbert et al., 1997). Gelatinase activity was significantly elevated in fluids from eyes of dogs with keratoconjunctivitis (Arican et al., 1999). More recently MMP-2 and MMP-9 were detected in canine osteosarcoma (Loukopoulos et al., 2004), cutaneous mast cell tumours (Leibman et al., 2000), mammary tumour (Santos et al., 2012), melanoma (Docampo et al., 2011), canine seminal plasma (Saengsoi et al., 2011), renal fibrosis (Aresu et al., 2011), myxomatous mitral valve disease (Ljungvall et al., 2011), visceral leishmaniasis (Marangoni et al., 2011), meningioma (Mandara et al. 2009), hemangiosarcomas (Murakami et al., 2009) and lymphoma (Gentilini et al.,2005).

1.2. VASCULAR ENDOTHELIAL GROWTH FACTOR

Vascular endothelial growth factor (VEGF) is usually considered an exclusive angiogenic growth factor. At least 6 VEGF types have been described: VEGF from A to E and placenta growth factor (PlGF). VEGF-A is the most common and most extensively studied isoform. VEGFs are glycosylated homodimers with sequence homology (20%) to A and B chains of PDGF and contain a core of cysteine residues. Three VEGF receptors—VEGRF-1 (flt-1), VEGFR-2 (KDR/flk-1), and VEGFR-3 (flt-4)—have been described. They belong to a subfamily of tyrosine kinase receptors within the PDGF receptor class and are characterized by 7 extracellular immunoglobulin-like domains. They are expressed in endothelial cells from embryogenesis. All VEGF isoforms can bind to VEGFR-1 and VEGFR-2. VEGFR-1 and VEGFR-2 are localized to vascular and lymphatic endothelial cells in adult tissues. VEGFR-3 is present mostly in the lymphatic endothelium. However, VEGFR-2 is the main VEGF receptor in endothelial cells (Halper, 2010).

1.2.1. REGULATION

The activity of tyrosine kinase receptors is regulated by the availability of ligands. A particular feature of the VEGF-A ligand is the upregulation of its expression levels under hypoxic conditions. Hypoxia allows the stabilization of hypoxia-inducible factors (HIFs) that bind to specific promoter elements which are present in the promoter region of VEGF-A. Similarly, expression of VEGFR-1 is directly regulated by HIFs. VEGFR-2 is also upregulated during hypoxia, but the role of different HIFs in its regulation remains to be clarified. VEGFR-3 expression is upregulated in differentiating embryonic stem cells that are cultured in a hypoxic atmosphere. After the binding of ligands, the VEGFRs are able to form both homodimers and heterodimers. The signal-transduction properties of the VEGFR heterodimers compared with homodimers remain to be elucidated. Dimerization of receptors is accompanied by activation of the receptor-kinase activity that leads to the autophosphorylation of the receptors. Phosphorylated receptors recruit interacting proteins and induce the activation of signalling pathways that involve an array of second messengers (Olsson et al., 2006). Furthermore several growth factors, including TGF-β, PDGF, and inflammatory cytokines, such as IL-6, upregulate VEGF mRNA expression, suggesting that paracrine or autocrine release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment (Ferrara et al., 2003).

1.2.2. ANGIOGENESIS AND CANCER

As mentioned before, angiogenesis plays an important role not only in the tumour growth and its blood supply, but also in the tumour metastasis (Fig. 4). Vessels at the tumour periphery modify from normal nonproliferating host vessels to tumour vessels generated in response to VEGF, without some complicating influences such as tumour secreted proteases, necrosis, hypoxia, and increased interstitial pressure, present in the tumour core. During tumourigenesis, neoplastic lesions initially undergo an avascular growth phase, followed by a second event, that is characterized by the switch from the avascular to vascular phenotype, or “the angiogenic switch”. This initiates a cascade of events that results in the expansion of tumour volume and subsequent metastasis (Hoeben et al., 2004). Furthermore, according to this accepted hypothesis of angiogenic switch, the induction of angiogenesis and subsequent tumour progression is dependent on the balance of a multitude of angiogenesis activators or inhibitors in the tumour microenvironment. Important activators include VEGF, basic fibroblast growth factor (bFGF), PDGF, epidermal growth factor (EGF), granulocyte colony stimulating factor (G-CSF), interleukin-1, interleukin-6, interleukin-8, and tumour necrosis factor α (TNFα). Whereas multiple antiangiogenic molecules include cytokines such as interferon (IFN)-α and –γ, as well as several peptides generated by proteolytic cleavage of the basement membrane and the ECM operated by MMPs. Another antiangiogenic pathway is through the release of soluble VEGF receptor-1 (sVEGFR-1) (Moehler et al., 2003). Molecular regulations of the angiogenic switch that involve VEGF are several. These regulations include:

- hypoxia-induced upregulation of VEGF;
- upregulation of VEGF after oncogenic transformation (mutant Ras, c-myc, myb, v-scr, nox-1);
- tumour and vascular cell interactions through adhesion molecules (IL-6 and CD40) and paracrine stimulatory circuit;
- remodelling ECM by MMPs and consequently release of angiogenic factors bound to ECM;
- bone marrow derived endothelial precursor cells;
- lymphangiogenesis with the expression of VEGF-C (Moehler et al., 2003).

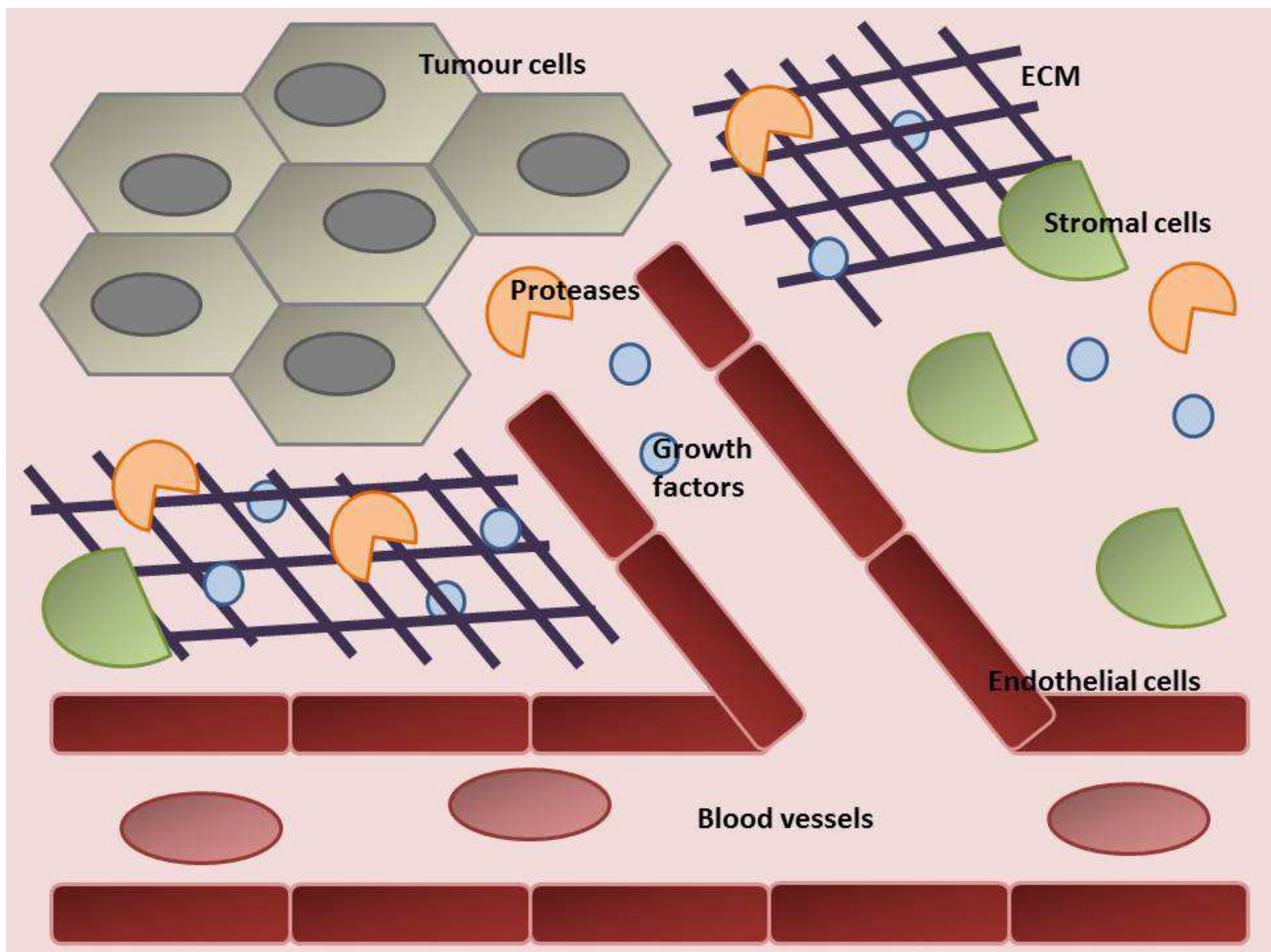

Fig. 4: Role of VEGF in tumour angiogenesis. Tumour cells of primary tumour release VEGF that binds to its receptors into nearby tissues on the endothelial cells of pre-existing blood vessels, leading to their activation. Crosstalk between cancer cells and cells of microenvironment leads to the secretion and activation of various proteolytic enzymes, such as MMPs, which degrade the basement membrane and ECM, permitting deposition of new basement membrane by endothelial cells. Simultaneously other growth factors, such as PDGF, are secreted to attract supporting cells (stromal cells) to stabilize new vessels. Integrin molecules, such as $\alpha_v\beta_3$-integrin, help to pull the sprouting new blood vessels forward.

1.2.3. TARGETED THERAPY IN HUMAN

Most of the angiogenesis inhibitors that are currently used for the treatment of cancer achieve their effects by blocking VEGF. Inhibitors of VEGF not only stop angiogenesis and destroy part of the tumour vasculature through apoptosis, but they also normalize tumour vessels. Through rapid and robust effects on the tumour vasculature, angiogenesis inhibitors slow the growth of many primary tumours and metastasis. Angiogenesis inhibitors have been approved for a wide range of

cancer types, including hepatocellular carcinoma and renal cell carcinoma that respond poorly to other agents (Sennino and McDonald, 2012). Bevacizumab, a function-blocking antibody to VEGF, is approved for use with chemotherapy to treat metastatic colorectal cancer, metastatic renal cell cancer, metastatic breast cancer and metastatic non-small-cell lung cancer; and as a single agent for recurrent glioblastoma and advanced ovarian cancer. Multiple other angiogenesis inhibitors that are approved for cancer therapy (such as sorafenib, sunitinib, axitinib, pazopanib and vandetanib) inhibit VEGF signalling by targeting receptor tyrosine kinases (Bhargava and Robinson, 2011). Disease progression, as reflected by tumour growth and metastasis during treatment with inhibitors of VEGF signalling, is attributed to multiple mechanisms. Among them are compensatory actions of angiogenic growth factors that are not blocked by inhibitors of VEGF signalling, blood flow alterations owing to tumour-vessel normalization, presence of normal peritumoural blood vessels, exaggeration of intratumoural hypoxia, activation of pathways that favour epithelial–mesenchymal transition (EMT), promotion of tumour invasiveness, suppression of immune surveillance, induction of tolerance and activation of cancer stem cells (Sennino and McDonald, 2012).

1.2.4. VEGF IN DOG

To date, little is known about VEGF and VEGFR expression in normal or pathological canine tissue. Scheidegger and colleagues (1999) demonstrated that canine and human VEGF are structurally almost identical, with the same biological and cell-binding properties, and that the canine VEGF receptors closely resemble their human counterparts. VEGF expression levels in dogs were investigated in mammary tumour (Santos et al., 2010), mast cell tumour (Rebuzzi et al., 2007), lymphoma (Zizzo et al., 2010), soft tissue sarcoma (Fernandes de Queiroz et al., 2012), congenital portosystemic shunt (Tivers et al., 2012), canine vascular tumour (Yonemaru et al., 2006), cutaneous fibrosarcoma (Al-Dissi et al., 2009), epithelial nasal tumour (Shiomitsu et al., 2009), osteosarcoma (Thamm et al., 2008), oral malignant melanoma (Taylor et al., 2007), cutaneous squamous cell carcinoma, trichoepithelioma (Al-Dissi et al., 2007) and primary intracranial neoplasms (Rossmeisl et al., 2007).

1.3. TRANSFORMING GROWTH FACTOR BETA

The transforming growth factor-β (TGF-β) superfamily of cytokines, which consists of TGFβs, activins, inhibins, bone morphogenetic proteins, anti-Müllerian hormone, as well as growth and differentiation factors, is conserved through evolution and found in all multicellular organisms. Three highly homologous isoforms of TGF-β exist in humans: TGF-β1, TGF-β2 and TGF-β3. They share a receptor complex and signal in similar ways but their expression levels vary depending on the tissue, and their functions are distinct. Proteolytic cleavage, interaction with integrins or pH changes in the local environment are known to activate latent TGF-β and free active TGF-β for binding to its receptors at the cell membrane (Shi et al., 2011).

1.3.1. REGULATION

Each TGF-β ligand is synthesized as a precursor, which forms a homodimer that interacts with its latency-associated peptide (LAP) and a latent TGF-β- binding protein (LTBP), forming a larger complex called the large latent complex (LLC). The TGF-β activation process involves the release of the LLC from the ECM, followed by further proteolysis of LAP to release active TGF-β to its receptors. MMP-2 and MMP-9 are known to cleave latent TGF-β. In addition to MMPs, thrombospondin 1 (THBS1) is known to activate latent TGF-β. Binding to the extracellular domains of type I and II receptors, TGF- β actives the intracellular serine/threonine kinase domains of the receptors, facilitating the phosphorylation and the subsequent propagation of signalling by SMAD-dependent pathway (Akhurst and Hata, 2012).

1.3.2. BIOLOGICAL ACTIONS

TGF-β is involved in a range of biological processes both during embryogenesis and in adult tissue homeostasis.

- *Inhibition of cell proliferation.* TGF-β strongly inhibits the growth of many cell types, including epithelial, endothelial, haematopoietic and immune cells; moreover it has pro-apoptotic and differentiation-inducing actions on epithelial cells. Together, these actions result in the context of tumour suppression. In oncology, many tumours are characterized by TGF-β growth-inhibitory effects, but this depends on the tumour type and the stage of tumour progression. In fact TGF-β may provide potent tumour-suppressive or tumour-

promoting functions directly on the tumour cell, presumably by mediating differential gene expression programmes (Heldin et al., 2009).

- *Induction of epithelial–mesenchymal transition (EMT) and the myofibroblast phenotype.* TGF-β can induce mesenchymal transition of both epithelial and endothelial cells. This has consequences for disease progression in both cancer and fibrosis. EMT increases cellular migration and invasive properties, as cell migration requires loss of cell–cell contacts and acquisition of fibroblastic characteristics. In breast and skin cancer, tumour cell EMT contributes to cancer progression as cells consequently become more migratory and invasive, and they can ultimately transition to a myofibroblastic phenotype, which leads to increase ECM elaboration and tissue contraction process (Derynck and Miyazono, 2008).
- *Extracellular matrix regulation.* As TGF-β is widely documented to increase collagen synthesis and deposition by fibroblasts, TGF-β has become a central therapeutic target for different types of fibrosis. TGF-β activity and the synthesis of ECM proteins are regulated by several genes known to be important in driving fibrosis; these genes are directly regulated by TGF-β–SMAD signalling pathways. There is a reciprocal regulation of TGF-β by the ECM: latent TGF-β, bound to ECM components, such as fibronectin and fibrillin, is inactive until physiological or pathological processes initiate its release (Akhurst and Hata, 2012).
- *Immune-suppression and inflammation*. TGF-β has potent growth-suppressing activity on most precursor cells of the immune system, particularly T and B cells. TGF-β is a potent suppressor of T cell proliferation and an inducer of B cell apoptosis. Additionally, the ligand can alter the course of immune cell differentiation. During tumour progression, excess TGF-β suppresses immune surveillance by attenuating the anti-tumour functions of CD8+ T cells, CD4+ T cells and dendritic cells (Flavell et al., 2010) (Fig. 5).

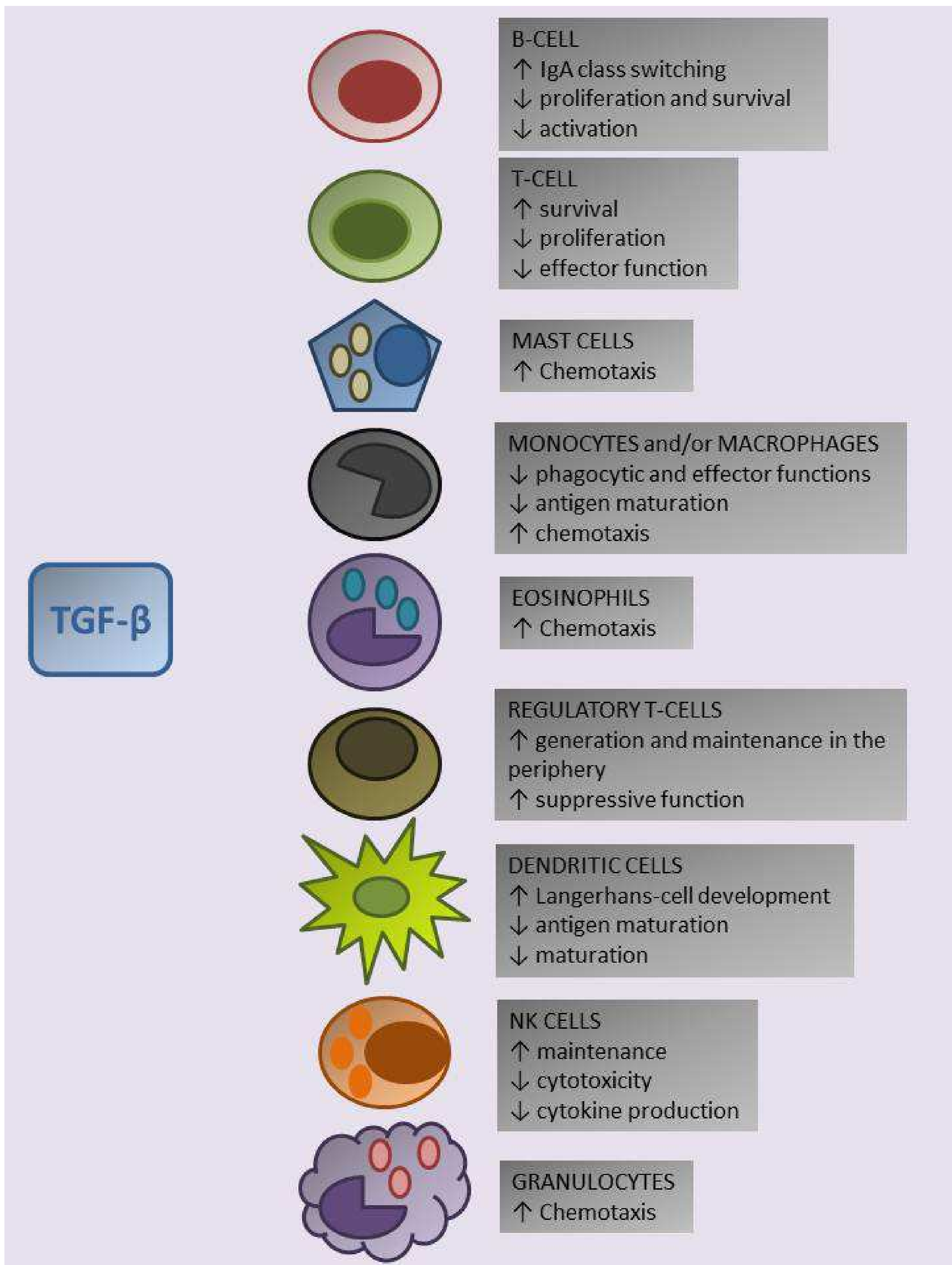

Fig. 5: TGFβ signalling on leukocytes. Transforming growth factor-β (TGF-β) signalling can affect leukocytes with positive or negative effects. NK, natural killer.

1.3.3. TARGETED THERAPY IN HUMAN

TGF-β has a biphasic action during tumourigenesis, suppressing it at early stages but promoting late-stage tumour progression. This is a paradigm for the action of TGF-β during disease progression in general, including that of fibrosis, inflammation and cardiovascular disease, and it derived by the fact that the normal function of this ligand is in the regulation of homeostasis. Therefor the main goal in cancer therapy is to downmodulate excessive levels of TGF-β ligands. In mouse gene knockout studies, two major concerns in TGF-β drug development against cancer have been the inadvertent inhibition of the tumour-suppressing function of TGF-β signalling and the development of adverse side effects unrelated to cancer, such as widespread inflammation, autoimmunity or cardiovascular defects (Larsson et al., 2001). Until now most clinical trials in oncology regard breast cancer, followed by glioblastoma. TGF-β signalling inhibitors are generally safe and may be efficacious in several clinical applications, especially in desperate cases such as end-stage cancer (Akhurst and Hata, 2012). Another objective regards cancer 'stem cells', or tumour-initiating cells. Several groups have now reported, in vitro and in vivo, that TGF-β induced EMT can drive tumour cells towards a more 'stem cell-like' phenotype (capacity to self-renew and to initiate and persistently propagate the entire tumour). It is important to note that, in cancer, the outcome of reduced TGF-β signalling may be highly dependent on the innate genetic background of the individual, especially when considering tumour microenvironment effects, such as immune surveillance (Akhurst and Hata, 2012).

1.3.4. TGF-β IN DOG

Little is known about TGF-β in canine tumours and one study shows that TGF-β activity was high in the tumour-bearing dogs (Itoh et al., 2009); another one gives a possible role of tumour suppressor for TGF-β in the development of hepatocellular carcinoma in dogs (Grabarević et al., 2009) and Klopfleisch and his colleagues (2010) suggest that loss of TGF-β-3 may have growth-stimulatory effects in late-stage tumours, and loss of its expression, together with reduced TGFβR-3 expression, may be associated with increased proliferative activity of canine mammary tumour similar to findings in human breast cancer.

1.4. PLATELET-DERIVED GROWTH FACTOR

Members of the platelet-derived growth factor (PDGF) family are major mitogens for connective tissue cells, glial cells, and certain other cell types. Structurally they are homodimers of related A-, B-,C-, and D-polypeptide chains, and an AB heterodimer. PDGF isoforms exert their cellular effects by binding to α- and β-tyrosine kinase receptors (Fig. 6). PDGF was originally purified from human platelets. Its isoforms stimulate proliferation, survival, chemotaxis, and differentiation of cells. They have important functions during embryogenesis and in the adult during wound healing and in the control of interstitial fluid pressure. Over-expression of PDGF has been linked to several pathological conditions, including tumours and other conditions involving an excess cell proliferation, such as fibrotic conditions and atherosclerosis (Heldin and Westermark, 1999).

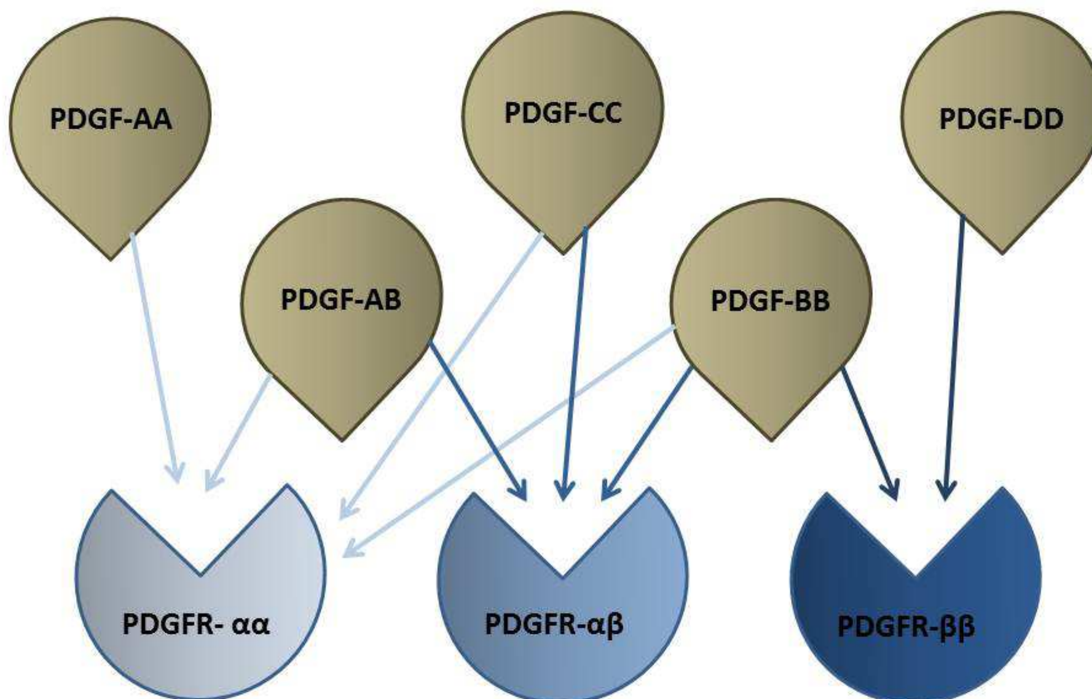

Fig. 6. The preferences of PDGF family members for PDGFRs. There are two receptor subunits that are homo- or hetero-dimerized. PDGF-BB is the universal ligand. PDGF-AB and PDGF-CC assemble and activate the PDGFR-αα and PDGFR-αβ. PDGF-DD activates the PDGFRββ, and under certain circumstances the PDGFRαβ. PDGF-AA is the most selective member of the PDGF family and activates PDGFR-αα exclusively.

1.4.1. REGULATION

Ligand binding induces dimerization of the receptors; the α-receptor binds all PDGF chains except the D-chain, whereas the β-receptor binds the B- and D-chains. Thus, the different PDGF isoforms induce different homo- and heterodimeric complexes of α- and β-receptors. In the dimeric receptor complexes, receptors phosphorylate each other in trans positions on specific tyrosine residues; finally members of the STAT family of transcription factors bind to activate PDGF receptors (Heldin, 2012).

1.4.2. PDGF AND CANCER

PDGF is often over-expressed in neoplasia and contribute to the growth of certain tumour types as well as to cells of the microenvironment of solid tumours, such as pericytes and smooth muscle cells of vessels and of stromal fibroblasts. Certain tumour cells expressing PDGF receptors produce PDGF isoforms, which stimulate cell growth and survival in an autocrine manner (Heldin and Westermark, 1999). The levels of expression of PDGF ligands as well as receptors are higher in more malignant tumours, suggesting that autocrine and paracrine effects of PDGF increase with degree of malignancy. Gliomas are probably the tumour type in which PDGF autocrine mechanisms are most important: nearly 30% of human gliomas show over-activity of PDGF receptor signalling. PDGF has also been implicated in other tumour types: in fact co-expression of PDGF and PDGF receptors has been reported in an AIDS-related Kaposi's sarcoma and in meningioma. Moreover, an autocrine PDGF-BB/PDGFR-β loop was found to mediate survival of large granular lymphocyte leukaemia of both T- and NK-cell origin (Yang et a., 2010).

In addition to classical autocrine stimulation, there are examples of mutations in the genes for PDGFRs, which cause their activation and promote tumourigenesis. Thus, in chronic myelomonocytic leukaemia (CMML), the kinase domain of PDGFR-β is fused to different partners. Analogously, in patients with idiopathic hypereosinophilia and systemic mastocytosis, the kinase domain of the PDGFR-α is fused to FIP1L1. In addition to the juxtaposition of the kinase domains of the receptors, the loss of regulatory sequences in the juxtamembrane and transmembrane domains is important for the increased autophosphorylation and initiation of signalling pathways promoting cell growth and survival. A majority of gastrointestinal stromal tumours (GISTs) have activating point mutations in Kit, a tyrosine kinase receptor for stem cell factor, which is structurally similar to PDGFRs (Heldin, 2012).

Epithelial cells normally do not contain PDGFRs. However, epithelial tumours can undergo EMT, changing in phenotype and making the tumour cells more invasive. In conjunction with EMT, PDGF and its receptors are induced. Interestingly, the metastatic potential of mammary epithelial tumours was shown to be dependent on an autocrine PDGF/PDGFRs loop. There are examples of stromal cells, such as smooth muscle cells, endothelial cells, and macrophages, which both can express PDGFRs and produce PDGF. However, it is not clear whether normal cells express PDGFRs and synthesize PDGF at the same time (Heldin, 2012).

1.4.3. TARGETED THERAPY IN HUMAN

In the last decades, the research in the field of tumour angiogenesis led to the development of a class of agents providing an effective inhibition of neovessels formation, through the blockade of VEGF-related pathways. More recently, the identification of several non-VEGF factors, such as PDGF, involved in tumour angiogenesis, in association with tumour progression, have emphasized the need to develop agents targeting multiple pro-angiogenic pathways. Besides the successful development of anti-VEGF drugs, novel agents targeting alternative angiogenesis-related pathways are being tested. Although it seems that the potential clinical usefulness of these novel compounds have been not yet fully investigated, sunitinib, sorafenib, pazopanib and other multikinase inhibitors, used in first line for advanced renal cell carcinoma, have certainly displayed encouraging results (Sennino and McDonald, 2012).

1.4.4. PDGF IN DOG

In veterinary oncology, PDGF has been investigated only in spontaneous canine astrocytomas, where increased PDGFRα expression was observed (Higgins et al., 2010); in canine osteosarcoma (Maniscalco et al., 2012), spontaneous canine hemangiosarcoma and cutaneous hemangioma (Asa et al., 2012).

2. AIM

It is evident from the data presented in this background that primary tumours and their metastasis are complex interactions consisting of numerous cell types. In analysing tumours, the experiments are usually focused upon a single cell type or a single gene product within a cell. However, it is naive to think that individual cell types work in isolation within a complex system. Thus, a major area for advances in understanding the role of the microenvironment must incorporate a system biology approach in order to model these interactions and their evolution over time. Both individual and collective migration modes are presumably further regulated by heterologous interactions between tumour cells and reactive stromal cells of the tumour microenvironment. Cancer cells can alter their adjacent stroma to form a permissive and supportive environment for tumour angiogenesis, invasion and metastasis, and therefore tumour progression, producing a range of growth factors and proteases that modify this 'reactive' tumour stroma.

A more comprehensive understanding of the molecular basis of diversity and adaptation of cell migration is therefore required to efficiently target cancer-cell motility and invasion. Little data exists for the biological and clinical role of growth factors and proteinases involved in tumour progression in domestic species.

The aims of this present work are to define gene expression profiling and proteomic analysis of different growth factors (VEGF-TGF-β-PDGF) and matrix metalloproteinases. Their possible prognostic and predictive role, in association with their crosstalk, will be also analysed in four different canine tumours:

- mammary tumour
- cutaneous mast cell tumour
- lymphoma
- lymphoid leukaemia

A better understanding of the tumour environment affecting cancer progression should provide new targets for the isolation and destruction of cancer cells via interference with the crosstalk established between cancer cells, host cells, and their surrounding extracellular matrix.

3. PHASE 1: Matrix metalloproteinases and their inhibitors in canine mammary tumours[*]

[*]Adapted with the permission of "Biomed Central" from: Aresu L, Giantin M, Morello E, Vascellari M, Castagnaro M, Lopparelli R, Zancanella V, Granato A, Garbisa S, Aricò A, Bradaschia A, Mutinelli F, Dacasto M. Matrix metalloproteinases and their inhibitors in canine mammary tumours. BMC Vet Res. 2011 Jul 4;7:33. © 2011 BioMed Central Ltd. All rights reserved.

BACKGROUND

Mammary neoplasia is one of the most common tumours in dogs, and malignant types occur in approximately half of canine mammary tumours. Invasion and metastasis are typical features of carcinomas (Karayannopoulou et al., 2005). The mRNA expression of MMP-2, MMP-9, TIMP-1, TIMP-2 and TIMP-3 has been extensively studied in vivo and in vitro in various human tumours (Stetler-Stevenson et al., 1989; Urbanski et al., 1992; Muller et al., 1993; Bhuvarahamurthy et al., 2006). In veterinary medicine, mRNA expression of these genes has been used to study canine neoplasia (Takagi et al., 2005; Nakaichi et al.,2007) and other diseases (e.g., meningitis-arteritis, chronic valvular disease, and arthritis)(Muir et al., 2007; Aupperle et al., 2009; Schwartz et al., 2010), but their expression in canine mammary tumours has not been specifically documented. Evaluation of the activities of TIMP-1, TIMP-2 and TIMP-3 in canine mammary tumour samples by reverse zymography has shown that low activity can be correlated with a malignant phenotype (Kawai et al., 2006). Membrane type 1 MMP (MT1-MMP) was the first MT-MMP to be identified as a major physiological activator of pro-MMP-2 in humans (Davies et al., 1993). Studies of canine mammary tumours suggest that pro-MMP-2 activation requires the formation of a ternary complex that consists of the C-terminal domain of pro-MMP-2, TIMP-2 and MT1-MMP (Papparella et al., 2002). The important role of another MMP family member, MMP-13, has been demonstrated in breast cancer and colorectal cancer (Chang et al., 2009; Huang et al., 2010). In veterinary medicine, reports of MMP-13 expression are only available for inflammatory and degenerative diseases (House et al., 2007; Disatian et al., 2008). After the sequencing of the entire dog genome, microarray technology has been used to characterize different canine mammary cell lines, progestin-induced canine mammary hyperplasia and spontaneous mammary tumours (Rao et al., 2008; Klopfleisch et al., 2010; Król et al., 2010), but, in literature, no targeted gene expression profiling studies are available for spontaneous canine mammary tumours.

AIM

The aim of the first study is to analyse MMP-2, MMP-9, MMP-13, MT1-MMP and TIMP-2 expression at both the mRNA and protein levels in canine mammary tumours including simple adenomas and carcinomas. The enzymatic activities of MMP-2 and MMP-9 are quantified by gelatin-zymography of the same homogenized tumour tissues and plasma from selected patients. Gene expression is evaluated also for TIMP-1, TIMP-3 and RECK. Moreover, the stromal compartments in these tumours are specifically evaluated.

METHODS

Tissue sampling

Fresh tissue samples were obtained from 35 dogs that underwent surgery for mammary neoplasia. The dogs underwent surgery due to evident disease, and the explicit consent of the owner was obtained. Excised tumour lesions were immediately divided into aliquots and stored under diverse conditions for different analytical techniques. For RNA isolation, aliquots of approximately 100 mg were immersed in RNAlater® solution (Applied Biosystems, Foster City, CA) and stored at -20°C until use. For histological examination and immunohistochemistry, the tissue was formalin-fixed and paraffinembedded. For gelatin-zymography, aliquots of up to 100 mg were frozen at -20°C until use.

Gene expression

Total RNA was isolated from control mammary glands, which were obtained from pathogen-free adult Beagles generously provided by GlaxoSmithKline Manufacturer S.p.A. (Verona, Italy) that had been used for other experimental purposes and 35 pathological samples using TRIzol® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The samples were purified with a classical phenol-chloroform extraction step. The total RNA concentration and quality were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and by denaturing gel electrophoresis. First-strand cDNA was synthesized from total RNA using the High Capacity cDNA Transcription Kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The generated cDNA was used as the template for quantitative real-time RT-PCR (qRT-PCR) in a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) using standard PCR conditions. The qRT-PCR reactions consisted of 1X LightCycler 480® Probe Master (Roche Diagnostics, Basel, Switzerland), 300 or 600 nM forward and reverse primers (the primer combination and final concentrations were optimized during assay setup), 100 nM human Universal Probe Library (UPL) probe (Roche Diagnostics) and 5 ng cDNA. The primers and human UPL probes shown in Table 1 were designed using the UPL Assay Design Centre web service. Calibration curves using a four-fold serial dilution of a cDNA pool revealed PCR efficiencies close to two and error values less than 0.2. Canine transmembrane BAX inhibitor motif containing 4 (CGI-119) and Golgin a 1 (GOLGA1) were chosen as reference genes for the absence of pathological statedependent differences in mRNA expression (Rao et al., 2008). Their amplification efficiencies were approximately equal to that of the target genes; moreover, no statistically significant difference was observed in their expression profiles between healthy and pathological samples.

The ΔΔCt method (Livak and Schmittgen, 2001) was used for the relative quantification of mRNA. Relative quantification (RQ) values were ultimately expressed as the fold change, such as RQ sample/control group mean RQ ratio, assuming that the control mammary gland mean RQ was equal to 1. Statistical analysis of the gene expression data was performed using the Kruskal-Wallis test followed by Dunn's post test. The correlation analysis of the target gene mRNA data was performed using a Spearman nonparametric test. In both analyses, GraphPad InStat 2.01 software (San Diego, California, USA) was used, and a p value < 0.05 was considered significant. Finally, Grubbs' test was used to identify outliers.

Table 1: Primer sequences and human UPL probes used for qRT-PCR amplification

Genes	**Accession number**	**Primer sequence (5'-3')**	**Human UPL probe**
MMP2	[GenBank:XM_535300.2]	F: gggaccacggaagactatga	29
		R: atagtggacatggcggtctc	
MMP9	[GenBank:NM_001003219.1]	F: tgagaactaatctcactgacaagca	6
		R: gctcggccacttgagtgta	
MMP13	[GenBank:XM_536598.2]	F: ctcttcttctcgggaaacca	50
		R: gcctggggtagtctttatcca	
MT1-MMP	[GenBank:XM_843664.1]	F: gatctgaatgggaatgacatctt	76
		R: gatggccgagggatcatt	
TIMP1	[GenBank:NM_001003182.1]	F: cagggcctgtacctgtgc	112
		R: cctgatgacgatttgggagt	
TIMP2	[GenBank:NM_001003082.1]	F: atgagatcaagcagataaagatgttc	93
		R: ggaggaaggagccgtgtag	

TIMP3	[GenBank:XM_538410.2]	F: tgctgacaggccgcgt	14
		R: gcagttacagcccaggtga	
RECK	[GenBank:NM_001002985.1]	F: aaggggtgtctgtctggagat	97
		R: cccaatttgcaaccttgaac	
CGI-119	[GenBank:XM_531662.2]	F: tctacaatctaagagagatttcagcaa	15
		R: ttcctgacaagcacaaaatcc	
GOLGA-1	[GenBank:XM_537849.2]	F: ggtggctcaggaagttcaga	149
		R: tatacggctgctctcctggt	

Immunohistochemistry

To analyse the expression of the MMP-2, MMP-9, MMP-13, MT1-MMP and TIMP-2 proteins by immunohistochemistry, contiguous 4 μm sections were cut from blocks of formalin-fixed, paraffin-embedded tissue, and the sections were placed on charged slides. After deparaffinization, the primary antibody incubation step for all antibodies was performed by an automated system (Ventana Medical Systems, Tucson, AZ). The pertinent antibody details are summarized in Table 2. The remainder of the staining procedure included incubation with a biotinylated anti-mouse secondary antibody, the diaminobenzidine substrate and a haematoxylin counterstain was performed using the Ventana ES automated immunohistochemistry system. Negative control slides were incubated with isotype-matched immunoglobulin in parallel with each staining batch to confirm the specificity of the antibodies. For each antibody, cases were semi-quantified for each protein-stained area. The intensity and the percentage of immunoassayed tumour cells were analysed. Each slide was scanned with a 400 × power objective in ten fields per slide, and the fields were selected by searching for protein-stained areas. A section was considered negative or positive according to the absence or presence of cytoplasmic staining. An intensity score of 0 was given if no staining was detected, 1 if there was weak to moderate staining, 2 if moderate to strong staining was present, and 3 if strong staining was detected. A total score for each examined field was obtained by multiplying the intensity score by the percentage of immunoassayed cells. A final ratio was obtained after averaging the ten selected fields. An image analysis system that

consisted of an Olympus BX51 microscope and software analysis (analySIS, Soft imaging system, Münster, Germany) was used. Furthermore, the immunoreactivity of each antibody was separately recorded for tumour and stromal cells in ten fields (400× power objective), and the final result was expressed as the average percentage from ten fields. For statistical analysis, dog tumours were subdivided into two groups: benign and malignant. Immunostaining score values for each protein were expressed as a median (range). The Mann-Whitney test was used to compare the immunostaining scores. Differences in percentages were calculated with the Chisquare test. The program SPSS 17.00 (SPSS Inc, Chicago, IL, USA) was used for all calculations.

Table 2: Details of antibodies

Antigen	**Source**	**Clone**	**Dilution**	**Manufacturer**
MMP-9	Human	MAB 3309	1:1000	Chemicon (Millipore)
MMP-2	Human	Ab-7	1:400	Neomarkers, Fremont, USA
TIMP-2	Human	MAB 3317	1:1000	Chemicon (Millipore)
MMP-13	Human	VIIIA2	1:100	Millipore Co., Billerica, USA
MT1-MMP	Human	-	1:200	Millipore Co., Billerica, USA

Zymography

MMP-2 and MMP-9 activity was studied by zymography, which reveals the gelatinase activity of latent proenzymes (zymogens) and mature MMPs. The homogenized tissue was centrifuged at 1500 rpm for 10 min, and the protein concentration of the supernatant was measured. The sample protein concentration was adjusted to 1 mg/ml, and 5 µl was diluted 1:1 in sample buffer; the final 10 µl sample was subjected to electrophoresis on an 8% SDS-PAGE gel copolymerized with 0.1% gelatin. Following electrophoresis, the gel was incubated for 1 h at room temperature in a 2.5% Triton X-100 solution and then at 37°C for 16 h in 0.5 M Tris-HCl buffer, pH 7.4, with 10 mM CaCl2.

The gels were stained with 0.1% Coomassie Brilliant Blue R-250 and de-stained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie- stained gelatin. Culture medium conditioned by A2058 melanoma cells was used as a control to identify the pro-MMP-9 gelatinolytic band, while conditioned media from HT1080 fibrosarcoma cells was used for the active forms of MMP-2 and MMP-9 and small amounts of the pro-MMP-2 (Davies et al., 1993). The amount of MMP-9 in 20 µl of the A2058 melanoma cell-conditioned media was defined as 100 arbitrary units (a.u.). The MMP-2 activity in 20 µl of HT1080 fibrosarcoma cell-conditioned media was defined as 100 a.u. The bands were quantified using an image analyser system that consisted of a GelDoc 2000 and Quantity One software (BioRad, Hercules, CA, USA). Peripheral blood samples were collected from 14 dogs 24 h before surgery. The plasma was obtained by blood sample centrifugation and stored at -20°C, and 10 µl was used for analysis. The plasma concentrations of MMP-2 and MMP-9 were evaluated by gelatin-zymography. The gelatinolytic activity was defined as the arbitrary optical density value of the sample relative to the optical density value of the control plasma from healthy dogs.

RESULTS

This study included 35 dogs with a median age of 9.9 years. Of the 35 tumours, 13 were simple adenomas, and 22 were simple carcinomas, according to the WHO classification system for canine mammary tumours (Misdorp, 2002).

Gene expression

The mRNA expression of MMP-2, MMP-9, MMP-13, MT1-MMP, TIMP-1, TIMP-2, TIMP-3 and RECK in mammary tumours and healthy mammary glands was investigated using a qRT-PCR approach. The overall results are reported in Table 3. All of the target genes were expressed in the control and tumour samples, with the exception of MMP-13, which was weakly expressed or not amplified in approximately half of the tumour samples (16 of 35 dogs). No statistically significant differences were observed for MMPs and inhibitors, particularly between benign and malignant tumours. Spearman correlation analysis was performed to identify potential relationships between MMPs and their preferential inhibitors at the transcriptional level and between MMP-2 and its well-known specific activator (MT1-MMP). Positive correlations were observed between MMP-2 and MT1-MMP ($r = +0.52$, $p < 0.01$), MMP-2 and TIMP-2 ($r = +0.47$, $p < 0.01$), MT1-MMP and TIMP-2 ($r = +0.39$, $p < 0.05$), and MMP-9 and TIMP-1 ($r = +0.56$, $p < 0.001$).

Table 3: MMPs and inhibitors mRNA expression in control mammary glands, benign and malignant mammary tumours

Gene	Control	Benign	Malignant
MMP-2	1.00 ± 0.46	1.01 ± 0.18	0.76 ± 0.12
MMP-9	1.00 ± 0.30	9.95 ± 4.40	7.23 ± 1.58
MMP-13	1.00 ± 0.28	2.08 ± 0.70	1.27 ± 0.25
MT1-MMP	1.00 ± 0.24	13.26 ± 6.09	2.82 ± 0.64
TIMP-1	1.00 ± 0.17	4.96 ± 1.27	3.97 ± 0.66
TIMP-2	1.00 ± 0.34	0.49 ± 0.09	0.60 ± 0.09
TIMP-3	1.00 ± 0.35	4.16 ± 1.62	2.38 ± 0.65
RECK	1.00 ± 0.52	3.41 ± 1.77	0.51 ± 0.10

Data are expressed in –fold changes (arbitrary units) as mean ± SEM. Statistical analysis: Kruskal-Wallis test + Dunn's post test.

Immunohistochemistry

Immunohistochemical staining revealed that MMP-2 and MMP-9 were present in all of the tumours examined. The two MMPs were strongly localized in the cytoplasm of the tumour epithelial cells (Fig. 1a, b,c). There were significant differences in the immunohistochemical score values for these two markers in benign and malignant neoplasia (Table 4). An intense immunoreaction was especially evident for MMP-2 in carcinomas, while the difference in MMP-9 immunolabelling in adenomas and carcinomas was lower. The MT1-MMP protein was detected in 21 tumours, and its expression was higher in malignant tumours. Tissue leukocytes and plasma cells stained positive for MMP-2 and MMP-9 as well as for MT1-MMP, and it was considered a positive control. Immunohistochemical staining revealed that TIMP-2 and MMP-13 were more highly expressed in simple adenomas than in simple carcinomas (Fig. 1e, f). The magnitude of the immunohistochemical detection of MMP-2, MMP-9 and MT1-MMP varied in the fibroblasts of the

stromal compartment. The staining intensity for MMP-2 and MT1-MMP was stronger in the fibroblasts closest to the epithelial tumour cells in the malignant tumours (Fig. 1d), while immunolabelled fibroblasts by the two proteins were scattered in the adenomas (Table 5). The percentage of fibroblasts that were positive for MMP-9 staining was lower than the percentage of those positive for MMP-2 in carcinomas. The percentage of TIMP-2- and MMP-13-positive fibroblasts was higher in adenomas than in carcinomas. The immunohistochemical data are summarized in Tables 4 and 5.

Table 4: Immunostaining score values for MMPs and TIMP-2 expressed as median (range) in benign and malignant mammary tumours

	Benign	**Malignant**	***P***
MMP-2	58.5 (45.4-145.5)	179.4 (120.1-267.3)	0.002
MMP-9	78 (26.8-189)	101.1 (34.3-213.9)	0.01
MMP-13	84.2 (0-198.2)	45.3 (0-101.2)	0.005
MT1-MMP	65.3 (0-230.2)	132.1 (34.2-221.2)	0.002
TIMP-2	74.3 (21.2-201.2)	48.9 (0-136.6)	n.s.

n.s.= not significant

Table 5: Immunohistochemical score for epithelial tumour cells and fibroblasts expressed as average percentage from ten fields in benign and malignant mammary tumours

	Benign (%)	**Malignant (%)**	***P***
MMP-2			
Tumour cells	88.6	67.5	0.05
Fibroblasts	11.4	32.5	
MMP-9			
Tumour cells	84.1	89.4	0.002
Fibroblasts	15.9	10.6	
MMP-13			
Tumour cells	56.4	65.9	n.s.
Fibroblasts	43.6	34.1	
MT1-MMP			
Tumour cells	80.3	55.8	0.001
Fibroblasts	19.7	44.2	
TIMP-2			
Tumour cells	53.3	79.6	n.s.
Fibroblasts	46.7	20.4	

n.s.= not significant

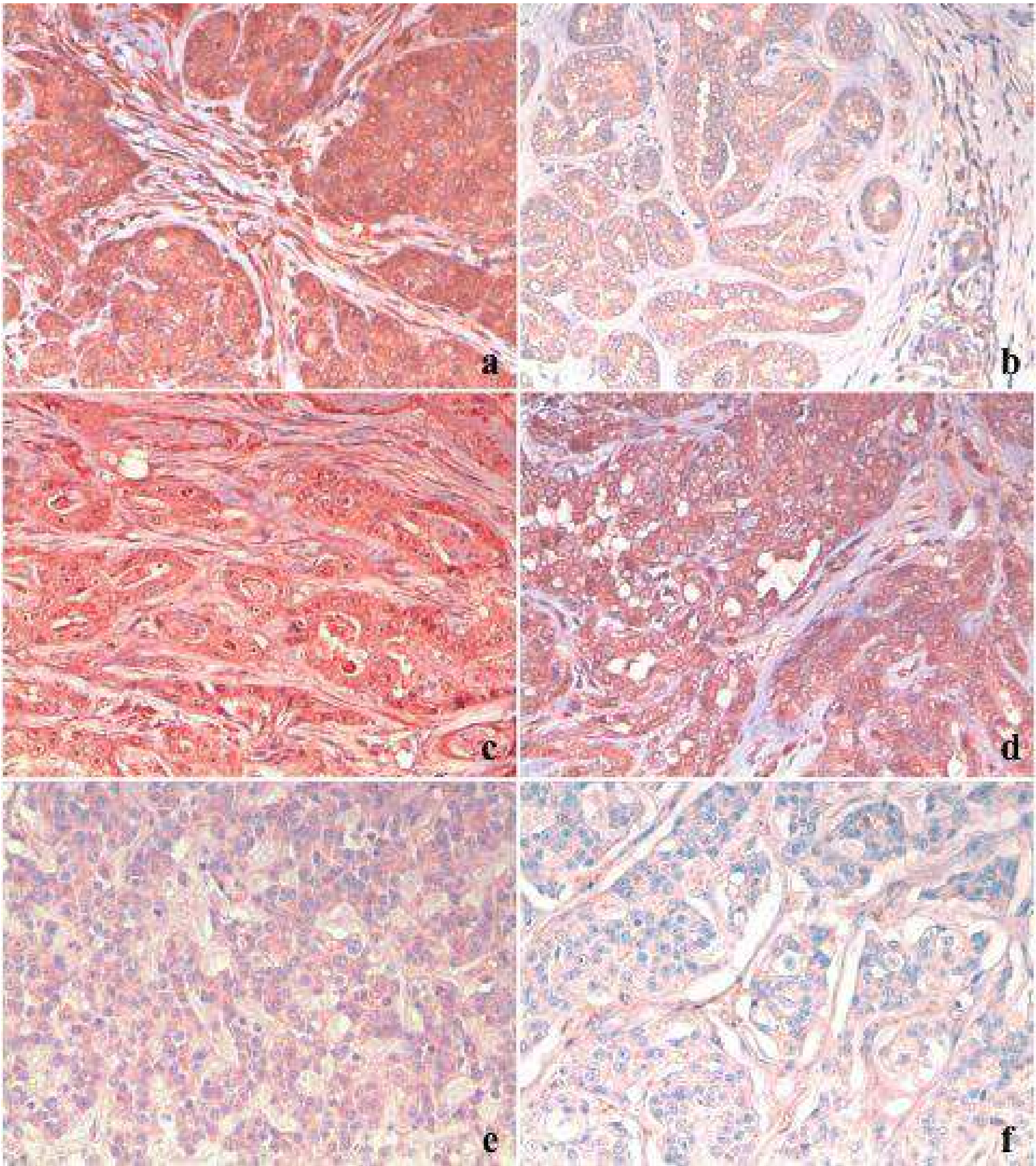

Fig. 1. Canine mammary tumours. (a) Epithelial tumour cells and stromal fibroblasts with intense MMP-2 immunopositivity in a carcinoma. (b) Epithelial tumour cells with weak MMP-2 immunopositivity in an adenoma. (c) Epithelial tumour cells and fibroblasts strongly MMP-9-immunolabelled in a carcinoma. (d) Epithelial tumour cells and stromal fibroblasts with intense MT1-MMP immunopositivity in a carcinoma. (e) Moderate TIMP-2 immunostaining in epithelial tumour cells in an adenoma. (f) TIMP-2 antibody-negative epithelial tumour cells in a carcinoma. (Immunohistochemistry, 200×).

Zymography

For each tumour sample, the active and inactive forms of MMP2 and MMP-9 were measured by their gelatinolytic activities (Fig. 2). The activities of MMP-2 and MMP- 9 were calculated in arbitrary units per 10 ng of protein. The pro-MMP-2 band was detected in all samples examined (adenoma and carcinoma). The activity of the carcinoma samples ranged between 16.7 and 59.9 units, while the activity of the adenoma samples ranged between 3.5 and 15.7 units. Bands for the active form of MMP-2 were found in 94% of the carcinoma samples and 17% of the benign tumour samples. In the simple carcinomas, the MMP-2 activity ranged between 47.4 and 87.5 units. The pro-MMP-9 band was expressed in all samples examined by gelatin-zymography. The activity range of the carcinoma samples varied greatly (from 7.5 to 106.4 units), while the activity range of the twelve benign samples was between 20.5 and 41.2 units. Only eight carcinomas exhibited bands for the active form of MMP-9. The activity was similar among the samples, ranging from 34.3 to 40.3 units. No bands were observed for the active form of MMP-9 in adenomas. The activities of both the pre- and activated forms of MMP-2 and MMP-9 were measured by gelatin-zymography of the plasma of fourteen dogs with mammary tumours and three healthy control dogs. A representative gel is shown in Fig. 3. Regardless of the tumour type, the band for pro-MMP-9 was significantly more evident in the plasma of dogs with tumours than in healthy dogs. The activated form of MMP-9 was not observed in healthy dogs but was present in all of the dogs with tumours. Pro-MMP-2 was detectable in the plasma of all dogs with tumours and also in control dogs with no difference in the level of expression, and the MMP-2 activated form was not detectable in either case.

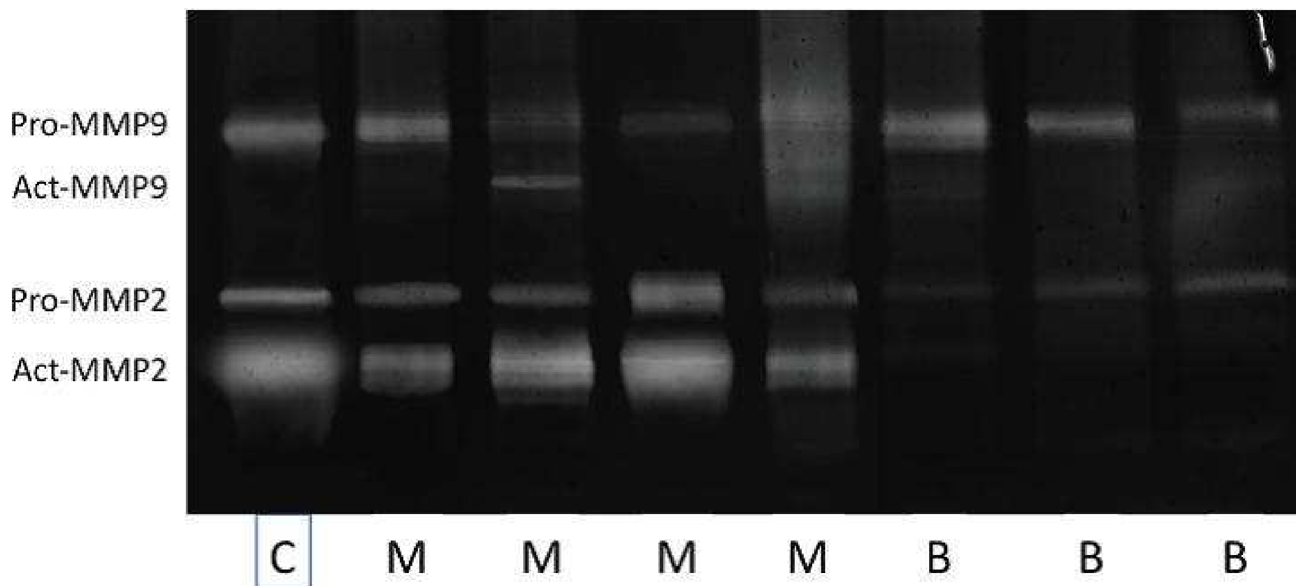

Fig. 2. Zymographic assay of gelatinase activity in malignant (M) mammary neoplasia, in benign (B) mammary neoplasia and in the control (C). Bands corresponding to latent and active forms of MMP-9 and to latent and active forms of MMP-2 were observed in carcinomas (M), while bands corresponding to latent forms of MMP-9 and MMP-2 were observed in adenomas (B). HT1080 fibrosarcoma cells (C) were used as the control for pro-MMP-9, pro-MMP-2 and active form of MMP-2.

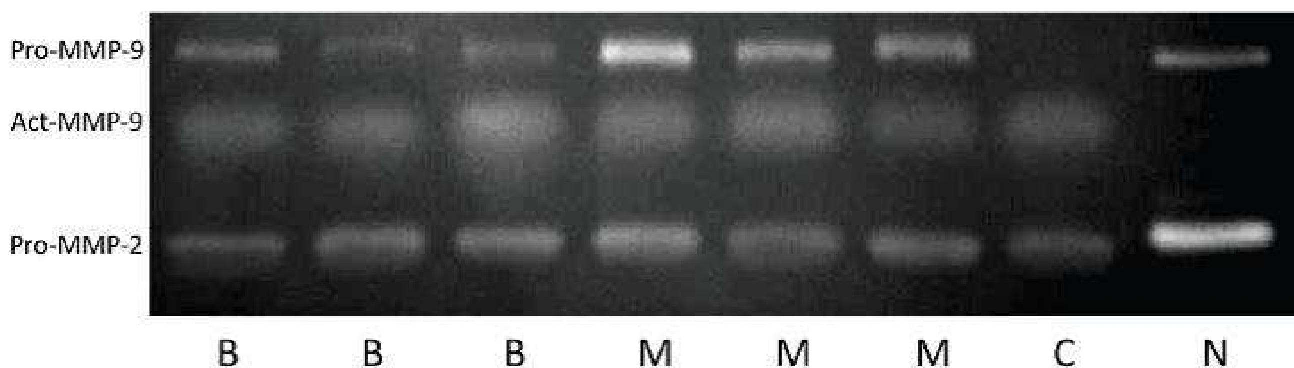

Fig. 3. Zymographic assay of gelatinase activity in pre-surgery plasma samples. (M = malignant mammary neoplasia, B = benign mammary neoplasia, C = control, N = normal dog). Bands corresponding to pro-MMP-2, pro-MMP-9 and active form of MMP-9 were observed in dogs with adenomas and carcinomas (B, M). Bands corresponding to pro-MMP-9 and pro-MMP-2 were observed in the control dog, and A2058 melanoma cells (C) were used as the control for active MMP-9.

DISCUSSION

In recent years, investigations of tumour growth have focused on the surrounding stroma in different tumours, considering important for cellular invasion, tumour growth and epithelial mesenchymal transformation (Bergamaschi et al., 2008; Zhang et al., 2009). The engagement of the neoplasia with a 'reactive stroma' provides structural and vascular support for tumour growth and also leads to tissue reorganization and invasiveness (Egeblad and Werb, 2002). MMPs play an important role in this mechanism by degrading the stromal connective tissue and basement membrane components. In addition, MMPs impact tumour cell behaviour in vivo because of their ability to cleave growth factors, cell adhesion molecules, and chemokines. The proteolytic activity of MMPs can be regulated at different levels, including gene expression, the conversion of the zymogen to the active enzyme, and by the presence of specific inhibitors. Among MMPs, MMP-2 and MMP-9 have mainly been associated with malignant tumour progression and metastasis in both human and canine tumours (Papparella et al., 2002; Nakaichi et al., 2007; Vizoso et al., 2007). In this study, the mRNA and protein levels of MMP-2, MMP-9, MMP-13, MT1-MMP and TIMP-2 in benign and malignant mammary tumours were thoroughly analysed. TIMP-1, TIMP-3 and RECK mRNA expression was analysed focusing on the expression of different MMP proteins in stromal fibroblasts to identify a possible role in canine mammary tumours. MMP-2 mRNA was expressed in all pathological samples, but no statistically significant differences were observed between benign and malignant tumours. On the contrary, at the protein level, immunohistochemistry identified higher MMP-2 expression in carcinomas; gelatin-zymography confirmed differences in MMP-2 production between adenomas and carcinomas ($p < 0.05$, data not shown). In particular, significant bands were observed for both the inactive and active forms of MMP-2 in simple carcinomas, while simple adenomas were characterized by minimal bands that corresponded to pro-MMP-2, suggesting that this enzyme is important during the infiltration process. Pro-MMP-2 may be converted to the active form during the infiltration process in malignancy; intense gelatinolytic activity has been described as necessary for malignant transformation (Tokuraku et al., 1995). These data are comparable to those from human studies. In human lung and gastric carcinomas, the MMP-2 activation ratio is enhanced; in canine tumour studies, higher MMP-2 and MMP-9 levels were observed in tumours than in inflammation, in malignant tumours than in benign tumours, in sarcomas than in carcinomas and in the advancing edge of canine malignancies than in the centre of canine tumours (Loukopoulos et al., 2003). In this study a band corresponding to active MMP-2 was observed in only two adenomas, and histological examination

of these two samples showed an intense stromal reaction close to the tumour. Activation of the zymogen form of MMP-2 is a cell surface event that is mediated by members of the membrane-type subfamily of MMPs, and MT1-MMP is the first physiological activator of pro-MMP-2 (Lambert et al., 2004). In this study, the immunohistochemical data for MMP-2 and MT1-MMP were comparable for both adenomas and carcinomas. MT1-MMP was distributed similarly to MMP-2 in malignant tumours and was reduced in benign tumours. Despite the opposing results obtained for mRNA and protein expression in benign and malignant mammary tumours, a close relationship between MMP-2 and MT1-MMP was observed at the pre-transcriptional level. Interestingly, immunohistochemistry showed an intense reaction for the stromal fibroblast component, with a significant increase in MMP-2 (p = 0.05) and MT1-MMP (p = 0.001) expression in the fibroblasts associated with carcinoma. MT1-MMP expression generally correlates well with MMP-2 activation in various human cancers, suggesting that MT1-MMP plays an important role in cancer cell invasion (Guedez et al., 1998; Takagi et al., 2005). This presumably occurs through direct ECM cleavage by MT1- MMP and perhaps via MT1-MMP-mediated pro-MMP-2 activation. Both MMP-2 and MT1-MMP have previously been detected by immunohistochemistry in canine mammary carcinomas (Papparella et al., 2002). However, the results obtained here were similar, suggesting that peritumour stromal cells may be a possible source of MMP-2 and MT1-MMP and promoters of invasion. Further studies in different tumours or in vitro are needed to support this hypothesis. qRT-PCR analysis revealed a high level of mRNA expression for MMP-9 in both benign and malignant mammary tumours. Epithelial cell immunoreactivity with the anti-MMP-9 antibody was observed in all tumours. MMP-9 positivity was higher in malignant tumours than in adenomas, confirming previous observations in human breast cancer (Del Casar et al., 2009) and canine mammary tumours (Hirayama eta l., 2002). Immunohistochemistry also revealed that the stromal fibroblasts had a minimal capacity to synthesize MMP-9. The enzyme activity was analysed by gelatin zymography; active MMP-9 form was not observed in any of the simple adenomas, and only eight carcinomas had a band for the active MMP-9 form, while all of the examined tumours had variable pro-MMP-9 gelatinolytic activity ($p < 0.01$ data not shown). The limited presence of the active form of MMP-9 may indicate a minor role for this gelatinase within the tumour, and it appears to be of little prognostic or pathogenetic value, as observed in canine tumours (Hirayama et al., 2002). TIMP-2 plays a double role, as both inhibitor and activator of MMP-2. Surprisingly, high TIMP-1 and TIMP-2 mRNA levels can predict adverse prognosis and be correlated with tumour aggressiveness in several different human cancers, including breast cancer (Ring et al.,

1997; Vizoso et al., 2007). In the present study, neither the mRNA nor the protein levels of TIMP-2 expression correlated with malignancy. MMP-13 was also evaluated. At the pretranscriptional level, it was expressed only in half of pathological samples, and immunohistochemistry revealed a higher level of expression in benign tumours than in malignant tumours. MMP-13 expression has previously been evaluated only in human tumours, for which MMP-13 involvement in tumour invasiveness was unclear (Freije et al., 1994). MMP-2 and MMP-9 plasma levels have been reported to be elevated in patients with various types of cancer. Plasma gelatinolytic activity in dogs was estimated by gelatin zymography in this work. Pro-MMP-9 and pro-MMP-2 were significantly concentrated in the plasma of all of the dogs, and bands for the latent forms were present in both animals with adenoma and animals with carcinoma. Moreover, the active form of MMP-9 was present in the plasma of all of the dogs with tumours with no differences in concentration between benign and malignant neoplasia. These data could indicate that the plasma levels of active MMP-9 are consistent with a proteinase activity that is due to the presence of an “ongoing disease,” and MMP-9 plasma levels may be a feasible method for detecting neoplastic growth in dogs in the future. TIMP-1, TIMP-3 and RECK mRNA expression were also evaluated and no statistically significant differences were observed among the groups. Contrasting results are reported in literature: microarray studies in dogs have shown that TIMP-1 and TIMP-3 are inhibited in progestin-induced canine hyperplasia relative to normal mammary glands (Rao et al., 2009). Besides, a qRT-PCR study of RECK mRNA expression in various spontaneously developing canine tumours showed that expression levels were low in the majority of tumour tissues relative to normal tissues; however, in some neoplasia, RECK expression was higher than in the controls (Takagi et al., 2005). To exert its MMP inhibitory or activating role, TIMP-1 binds preferentially to MMP-9, and TIMP-2 binds to MMP-2 (Stetler-stevenson et al., 1989; Declerck et al., 1991). This relationship was confirmed in this study by the statistically significant correlations that were found between MMP-2 and TIMP-2, MMP-9 and TIMP-1 and MT1-MMP and TIMP-2 mRNA. The observed discrepancy between the mRNA and protein data has also been described by other studies (Caenazzo et al., 1998). Enzyme accumulation that is not accompanied by an increase in mRNA could be due to a feedback mechanism that shuts off mRNA expression after the secretion and/or binding of the protein. In addition, the samples used may not be representative (Caenazzo et al., 1998). Extracting and amplifying mRNA from cells obtained through the laser capture microdissection of the same formalin-fixed tissue that was used for immunohistochemistry might better clarify the mRNA/protein expression discrepancy observed in the present study. In

conclusion, the involvement of MMP-2, MT1-MMP, MMP-9 and various TIMPs was evaluated in canine mammary tumours, with an emphasis on the stromal compartment. The present work opens the possibility of developing new therapies and supports the use of the dog as an animal model in this field for studying the role of MMP-2 and MT1-MMP in the mechanism of cancer. MMPs may be target molecules of the switch mechanism that leads to the progression of carcinomas from adenomas.

4. PHASE 2: Expression of Matrix Metalloproteinases, Tissue Inhibitors of Metalloproteinases and Vascular Endothelial Growth Factor in Canine Mast Cell Tumours*

*Adapted with the permission of "Elsevier" from: Giantin M, Aresu L, Benali S, Aricò A, Morello EM, Martano M, Vascellari M, Castagnaro M, Lopparelli RM, Zancanella V, Granato A, Mutinelli F, Dacasto M. Expression of matrix metalloproteinases, tissue inhibitors of metalloproteinases and vascular endothelial growth factor in canine mast cell tumours. J Comp Pathol. 2012 Nov;147(4):419-29. doi: 10.1016/j.jcpa.2012.01.011. Epub 2012 Apr 19. © 2012 Elsevier Ltd. All rights reserved.

BACKGROUND

Mast cell tumours (MCTs) are the most common cutaneous tumour of the dog (Thamm and Vail, 2007) and are always considered potentially malignant, but their true metastatic potential is not entirely known (Welle et al., 2008). Currently, the prognostic significance of MCT is assigned through histological grading, but numerous studies have shown significant differences between well- and poorly-differentiated MCTs in terms of survival times and disease-free intervals. In particular, the prognosis for intermediate MCT (grade 2) is difficult to predict: the tumour might behave in a benign fashion or recur and metastasize. Well-differentiated tumours have a metastatic rate of <10%, intermediate tumours are considered low to moderate in metastatic potential and undifferentiated tumours have a much higher metastatic rate (55-96%; Welle et al., 2008). Uncontrolled cellular proliferation plays a significant role in the progression of canine MCTs (Sakai et al., 2002; Scase et al., 2006; Webster et al., 2007; Gil da Costa et al., 2007). Despite these issues, information about ECM degradation and vascular angiogenesis in canine cutaneous MCT is sparse. MMPs have been evaluated only at the catalytic activity level through gelatin zymography (GZ) in samples from grade 2 and 3 tumours (Leibman et al., 2000) and VEGF has been detected at the protein level by immunohistochemistry (IHC) (Mederle et al., 2010) and by enzyme linked immunosorbent assay (ELISA) analysis (Patruno et al., 2009).

AIM

Thus, in order to better understand the role of ECM degradation and angiogenesis in canine MCTs of different histological grades, the aim of the present study is to evaluate the mRNA and protein expression of MMP-2, MMP-9, MT1- MMP, TIMP-2 and VEGF-A in MCTs subdivided by histological grade (Patnaik et al., 1984). The catalytic activities of both latent and active forms of MMP-2 and MMP-9 are also evaluated.

METHODS

Case Selection and Tissue Sampling

Fresh tissue samples were obtained from 35 dogs that underwent surgery for MCT. The consent of the owners to use the tissues for research purposes was obtained. Only samples of suitable size and quality were included in the study. After excision of the entire tumour, multiple samples (50-

100 mg) were collected from the central core of the mass. For total RNA isolation, aliquots were immersed in RNAlater® solution (Applied Biosystems, Foster City, California, USA) and stored at -20°C until used. For GZ, aliquots were frozen at -20°C until used. The remaining tissue was formalin-fixed and paraffin wax-embedded for microscopical examination and IHC.

Gene Expression

Total RNA isolation, cDNA synthesis, primers, calibration curve data and the formulation of final value are reported in Phase 1. Canine VEGF-A (GenBank reference sequences: NM_001003175.2, NM_001110502.1 and NM_001110501.1) was amplified with 50-CGT GCC CAC TGA GGA GTT-30 (forward primer, 300 nM final concentration) and 50- GCC TTG ATG AGG TTT GAT CC-30 (reverse primer, 300 nM) and human Universal Probe Library (UPL) probe number 9 (100 nM), while canine MMP-2, MMP-9, MT1-MMP, TIMP-2 and the reference genes transmembrane Bcl-2-associated X protein inhibitor motif containing 4 (CGI-119) and golgin A1 (GOLGA-1) were amplified with the primer pairs and UPL probes reported in Aresu et al. (2011).

Immunohistochemistry

The details of the immunohistochemal performance, the antibodies and the labelling valuation are described in Phase 1. Also a polyclonal rabbit antiserum specific for VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA; diluted 1 in 200) was also used.

Gelatin Zymography

Gelatine zymography performance and the quantification of bands are reported in Phase 1.

Statistical Analysis

Statistical analysis of the gene expression and GZ data was performed using the non-parametric Kruskal-Wallis test followed by Dunn's post test. The correlation between target gene mRNA data and between mRNA and protein activity results was performed using a Spearman non-parametric test. For all of these analyses, GraphPad InStat 2.01 software (San Diego, California, USA) was used and a p value < 0.05 was considered significant. To compare the IHC scores, differences in percentages were calculated with the Chi-square test. The SPSS 17.00 (SPSS Inc., Chicago, Illinois, USA) programme was used.

RESULTS

Case Selection

The study included samples from 35 MCTs from 35 dogs with a median age of 7.9 ± 2.7 years. Of the 35 tumours, seven were grade 1, 22 were grade 2 and six were grade 3. Male (14; three neutered) and female (17; two neutered) dogs were included in the study and in four cases the gender was not recorded. The following breeds were represented: crossbred (10), boxer (6), Labrador retriever (5), dogo Argentino (2), golden retriever (2), American Staffordshire bull terrier (1), dachshund (1), beagle (1), Boston terrier (1), cocker spaniel (1), Dalmatian (1), dogue de Bordeaux (1), rottweiler (1), English setter (1) and Siberian husky (1).

Gene Expression

All of the target genes were expressed in each MCT sample (Table 1). Between tumour grades, significant differences were found only for TIMP-2 where less TIMP-2 mRNA was found in grade 3 compared with grade 2 tumours ($p < 0.05$). In contrast, there was a progressive, but not significant, increase in MMP-9 and VEGF-A expression with increasing histological grade. MMP-2 and its specific activator (MT1-MMP) showed the same gene expression profile ($r = +0.91$, $p < 0.0001$; Fig. 1). The MMP-2:TIMP-2 and MMP-9:TIMP-1 mRNA ratios were also determined. Progressive, but not significant, increases in both ratios were observed with increasing tumour grade (Fig. 2).

Table 1: MMP-2, MMP-9, MT1-MMP, TIMP-2 and VEGF-A mRNA expression

mRNA expression (a.u.)			
	Grade 1	**Grade 2**	**Grade 3**
MMP-2	2.54 ± 0.68	7.45 ± 3.50	2.03 ± 1.61
MMP-9	0.62 ± 0.20	1.27 ± 0.39	5.66 ± 3.67
MT1-MMP	1.19 ± 0.22	3.43 ± 1.42	1.40 ± 0.66
TIMP-2	1.14 ± 0.29	1.69 ± 0.25 [c]	0.54 ± 0.21
VEGF-A	0.86 ± 0.37	1.56 ± 0.42	1.92 ± 0.75

Data are expressed as the mean ± standard error.

a, b, c Significant differences between grade 1 and 2, grade 1 and 3 and grade 2 and 3, respectively (Kruskal-Wallis test followed by Dunn's post test; c: $p < 0.05$).

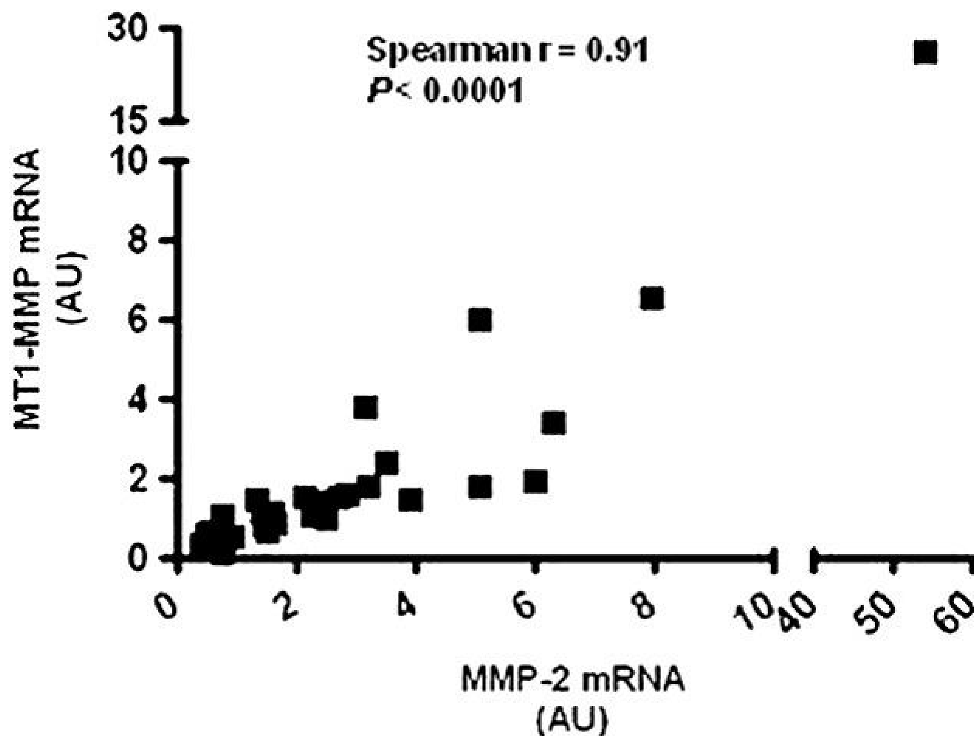

Fig. 1. Spearman correlation analysis between MMP-2 and MT1-MMP mRNA expression.

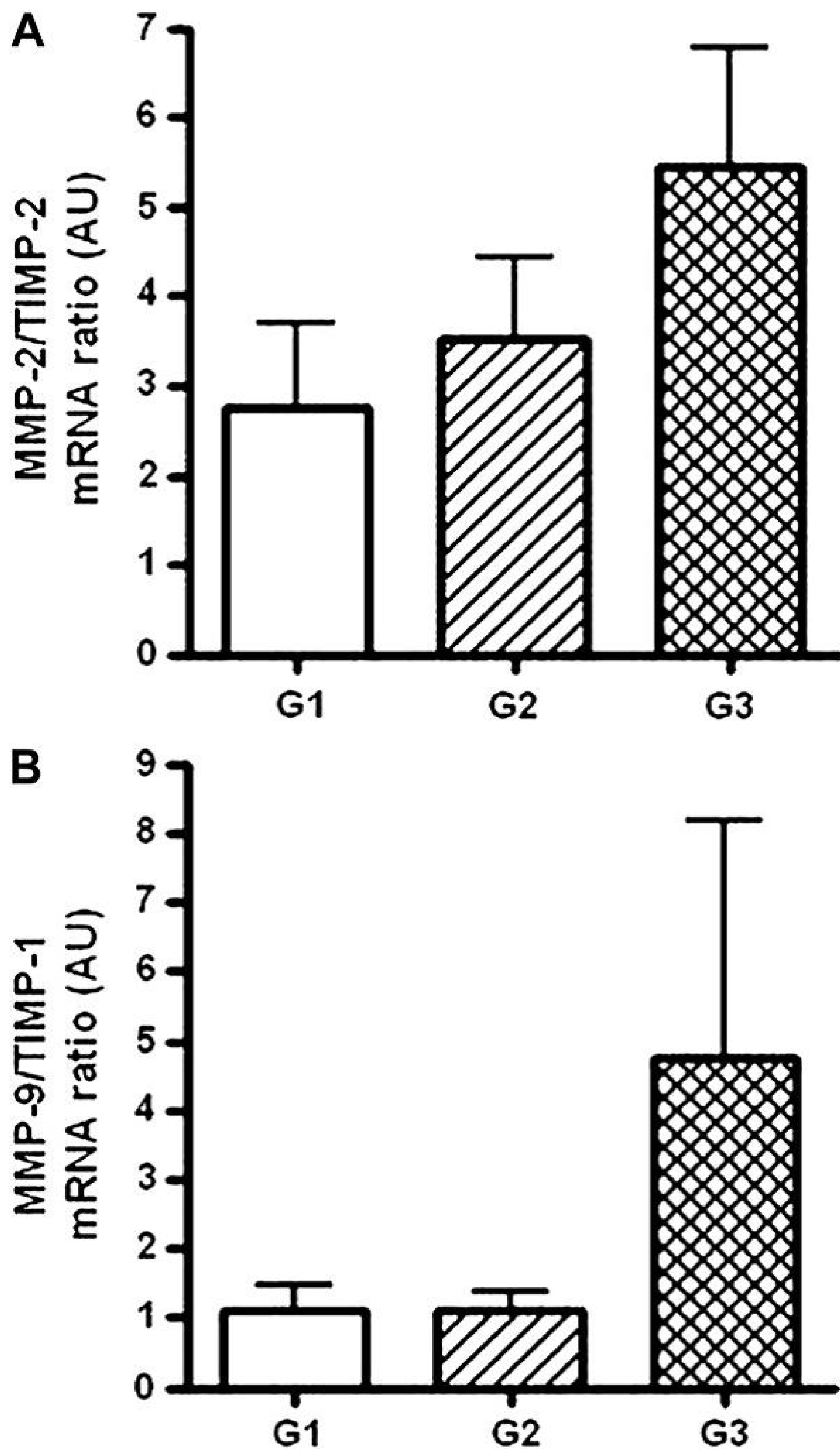

Fig. 2. (A) MMP-2:TIMP-2 and (B) MMP-9:TIMP-1 mRNA ratios in grade (G) 1, 2 and 3 MCTs. Data (mean± standard error) are expressed in a.u.. Statistical analysis: Kruskal-Wallis test followed by Dunn's post test.

Immunohistochemistry

MMP-2 and MMP-9 were present in all of the tumours examined (Table 2). Both molecules were localized to the cytoplasm of mast cells, while eosinophils appeared negative. MMP-9 expression was diffuse, while MMP-2 expression was multifocal (Fig. 3). Significant differences in the IHC scores were observed for MMP-9 between the three grades of MCT ($p < 0.05$). The most intense expression was in grade 3 MCTs (Fig. 4 and Fig. 5). The same behaviour was also observed for MMP-2, but this did not reach statistical significance (Fig. 3 and Fig. 5). MT1-MMP protein was observed in all tumours with cytoplasmic immunolabelling of few scattered neoplastic cells (data not shown). MT1-MMP expression increased with tumour grade without reaching statistical significance. In contrast, TIMP-2 expression was stronger in grade 1 than in grade 3 MCTs ($p < 0.01$) (Fig. 6 and Fig. 5). Finally, VEGF-A expression was stronger in grade 3 than in grade 1 MCTs ($p < 0.001$; Fig. 7 and Fig. 5). Labelling was mainly of the cytoplasm of neoplastic cells, in particular those cells adjacent to blood vessels.

Table 2: MMP-2, MMP-9, MT1-MMP, TIMP-2 and VEGF-A IHC

IHC score				
	Grade 1	**Grade 2**	**Grade 3**	**Significance**
MMP-2	34.1 ± 29.2	78.3 ± 39.1	159.0 ± 21.2	ns
MMP-9	91.9 ± 10.3	187.2 ± 54.3	279.3 ± 23.2	P < 0.05
MT1-MMP	45.3 ± 23.0	103.4 ± 25.2	183.3 ± 31.4	ns
TIMP-2	182.4 ± 12.2	143.2 ± 17.8	97.3 ± 10.3	P < 0.01
VEGF-A	106.6 ± 9.6	201.3 ± 15.5	291.3 ± 4.7	P < 0.001

ns, not significant.

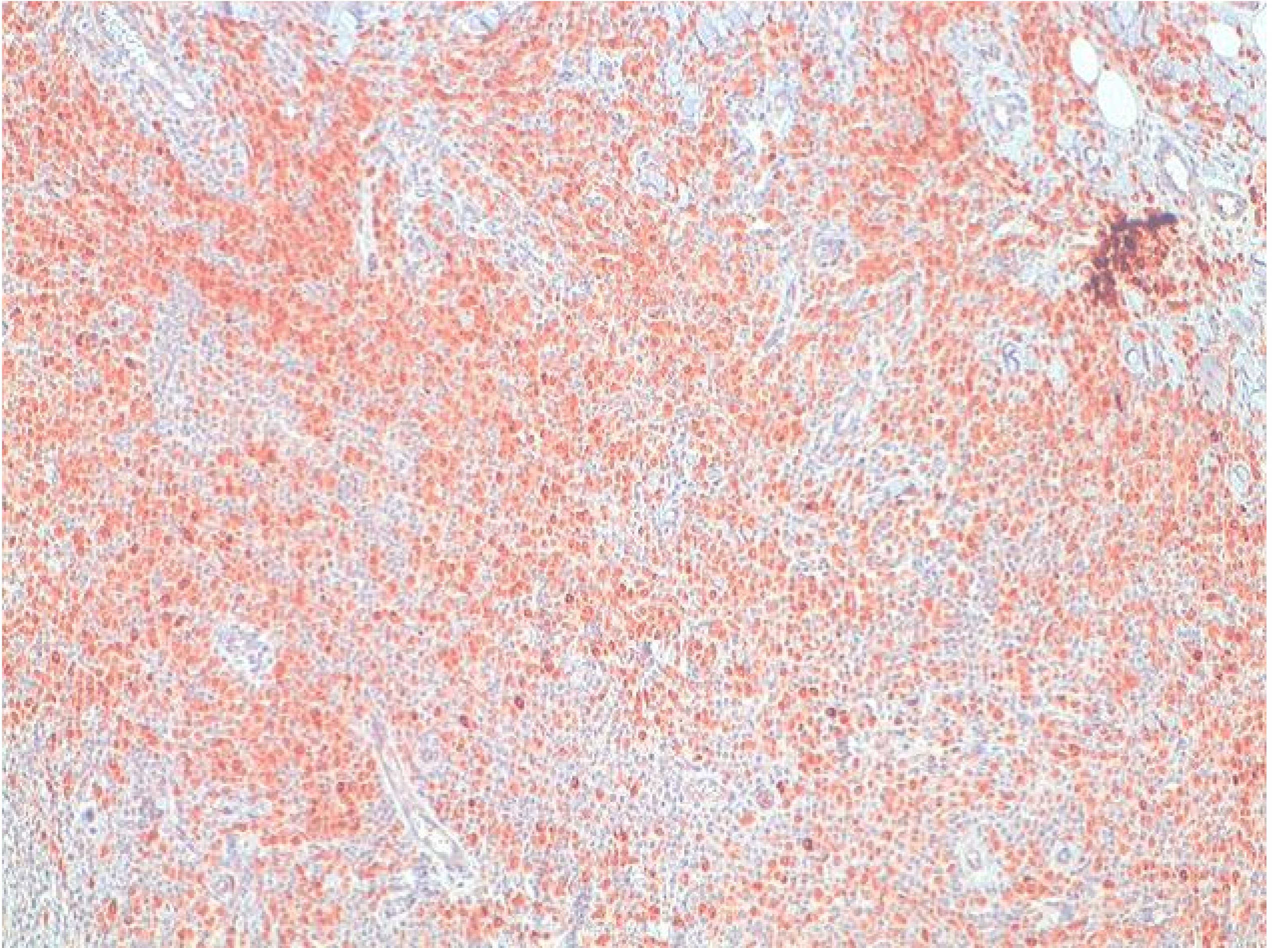

Fig. 3. Grade 3 MCT showing intense expression (multifocal to coalescing distribution) of MMP-2. IHC. X200.

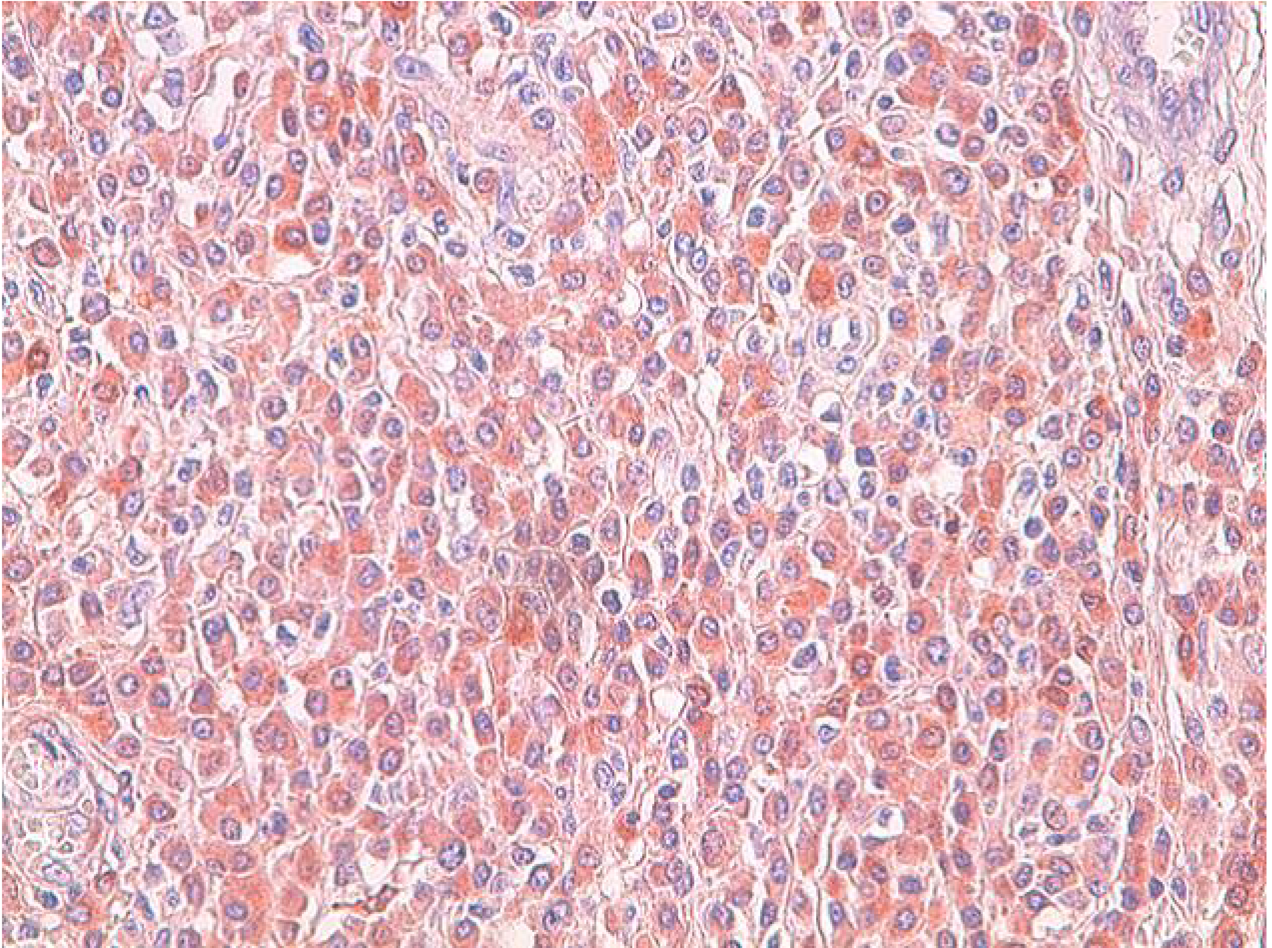

Fig. 4. Grade 3 MCT showing intense and diffuse expression of MMP-9. IHC. X400.

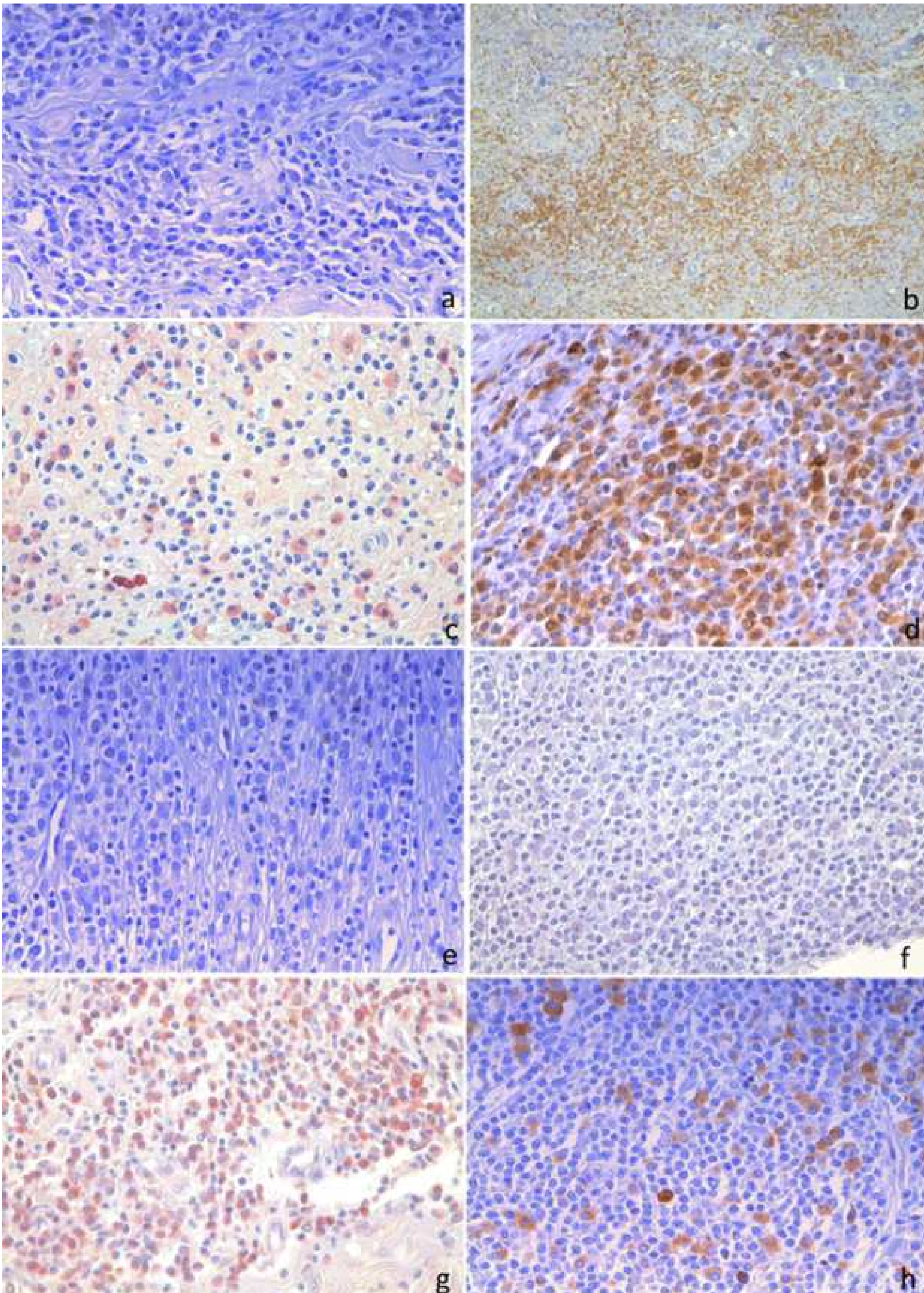

Fig. 5. (a) MCT grade I showing negative labelling of neoplastic cells to MMP-2 (×400); (b) MCT grade II showing moderate and multifocal expression of MMP-2 (×200); (c) MCT grade I showing weak expression in rare neoplastic mast cells of MMP-9 (×400); (d) MCT grade II showing intense and diffuse expression of TIMP-2 (×200); (e) MCT grade III showing negative mast cells immunolabelled for TIMP-2 (×400); (f) MCT grade I showing negative mast cells immunolabelled for VEGFR-2 (×400); (g) MCT grade II showing immunohistochemical labelling of neoplastic cells for VEGF (×400); (h) MCT grade I showing scattered immunohistochemical labelling of neoplastic cells for VEGF antigen (×400).

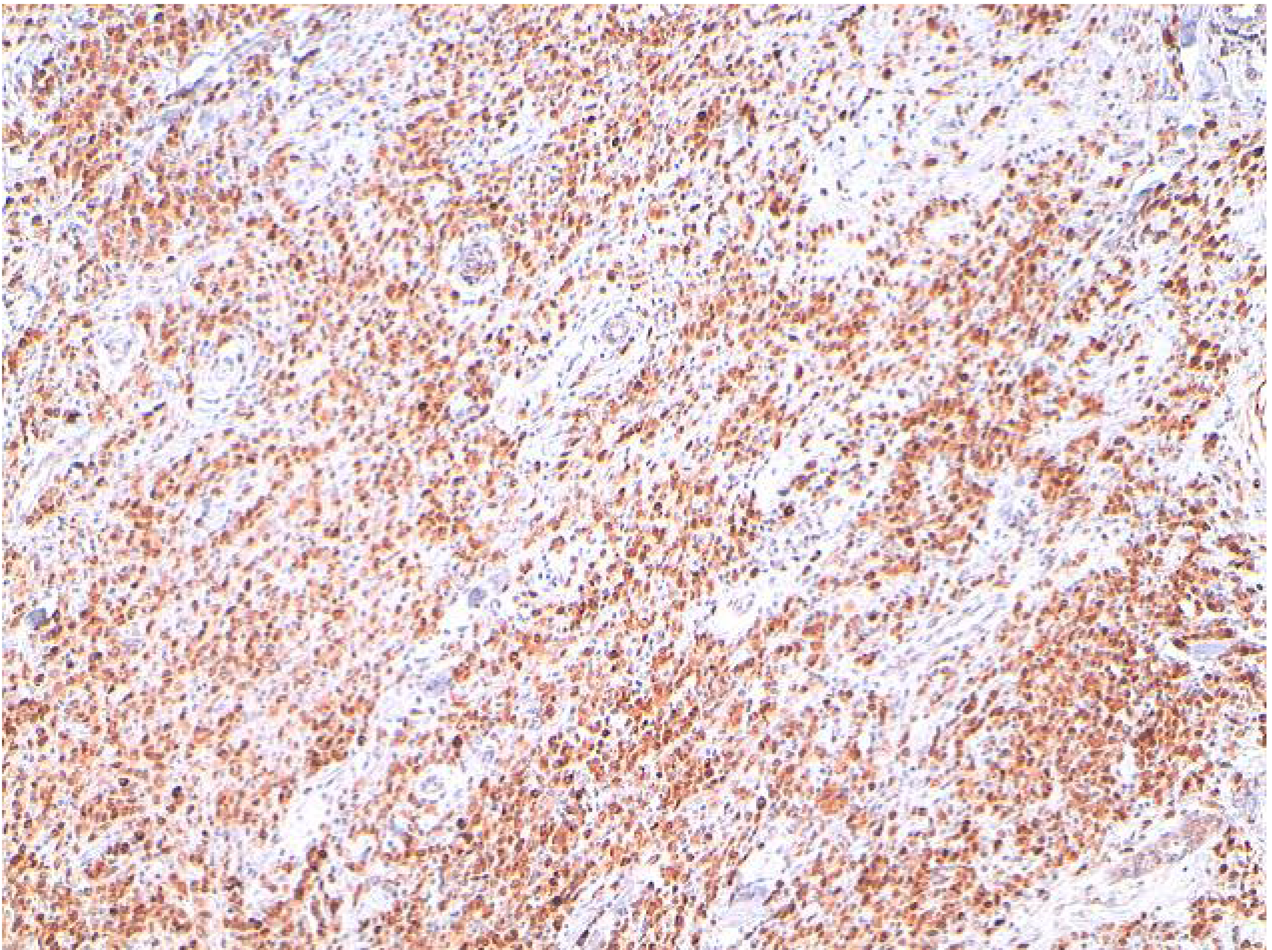

Fig. 6. Grade 1 MCT showing intense and diffuse expression of TIMP-2. IHC. X200.

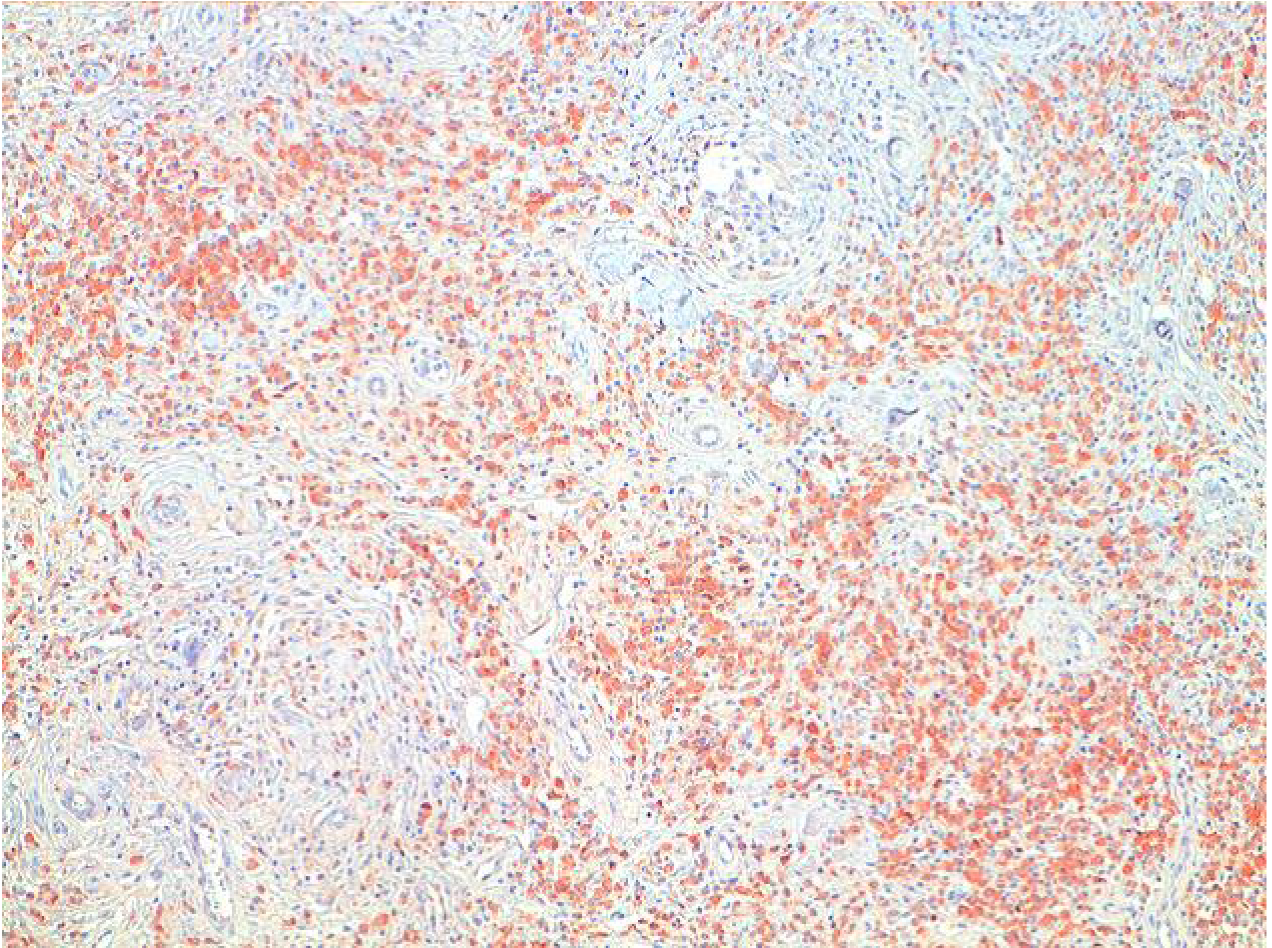

Fig. 7. Grade 3 MCT showing expression of VEGF antigen by tumour cells. IHC. X200.

Gelatin Zymography

Active and inactive forms of MMP-9 and MMP-2 were measured by GZ (Fig. 8). The activities of MMP-9 and MMP-2 were calculated in a.u. per 10 ng of protein. The pro-MMP-9 band was present in all samples examined by GZ. The activity range in grade 1 tumours was 2.3 - 60.2 a.u., in grade 2 tumours 71.0 - 134.3 a.u. and in grade 3 MCTs 151.2 - 218.9 a.u. Thirty-four tumours exhibited bands for the active form of MMP-9. The activity ranged from 0 to 40.3 a.u. for grade 1, 54.3 - 192.6 a.u. for grade 2 and 160.3 - 248.0 a.u. for grade 3 tumours. Pro- and active-MMP-9 activities progressively increased from grade 1 to 3 MCTs. Both were higher in grade 3 than in grade 2 ($p < 0.05$), in grade 3 than in grade 1 ($p < 0.001$) and in grade 2 than in grade 1 tumours ($p < 0.01$; Fig. 7B). Furthermore, the activity of the MMP-9 latent form was significantly higher than the active form ($p < 0.05$) in grade 2 and 3 MCTs. Densitometric values of the active form of MMP-9 were also correlated to MMP-9 mRNA expression (r = 0.37, $p < 0.05$). Bands for the latent and active forms of MMP-2 were found in 97% and 94% of MCTs, respectively. A progressive increase in MMP-2 gelatinolytic activity with histological grade was observed. The activity of pro-MMP-2 was 0-31.4 a.u., 28.7-164.0 a.u. and 65.3-149.7 a.u. in grade 1, 2 and 3 tumours, respectively. The activity of MMP-2 was 0-23.9 a.u., 13.2-169.7 a.u. and 70.8-161.2 a.u. in grade 1, 2 and 3 MCTs, respectively. Significant differences were obtained for pro-MMP-2 in grade 1 versus grade 2 and in grade 1 versus grade 3 ($p < 0.001$) and for active-MMP-2 in grade 1 versus grade 2 ($p < 0.01$) and in grade 1 versus grade 3 ($p < 0.001$) (Fig. 8C). Pro-MMP-2 activity was significantly higher than active-MMP-2 only in grade 1 samples ($p < 0.05$).

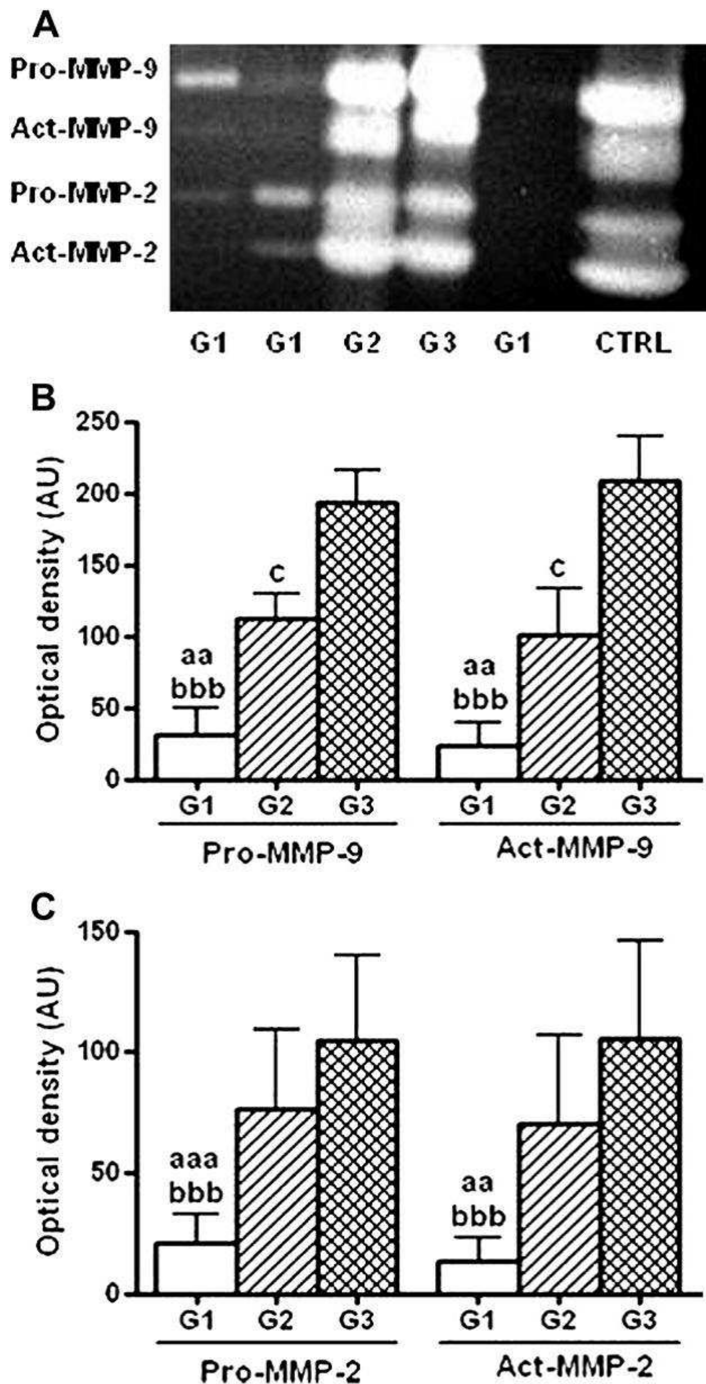

Fig. 8. (A) Zymographic assay of gelatinase activity in grade 1, 2 and 3 MCTs and in HT1080 fibrosarcoma cells (CTRL). Bands corresponding to both latent (pro-) and active (act-) forms of MMP-9 and MMP-2 were observed in MCTs. HT1080 fibrosarcoma cells were used as the control for pro-MMP-9, pro-MMP-2 and act-MMP-2. (B and C) Densitometric analysis of the bands corresponding to latent and active forms ofMMP-9 (B) andMMP-2 (C) in grade 1, 2 and 3 MCTs. Integrated density values for each group (mean _ standard deviation) are expressed as a.u. as a percentage of the density recorded in CTRL (100 a.u.). a, b, c Significant differences between grade 1 and 2, grade 1 and 3 and grade 2 and 3, respectively (Kruskal-Wallis test followed by Dunn's post test; c: $p< 0.05$; aa: $p < 0.01$; aaa, bbb: $p < 0.001$).

DISCUSSION

Dogs are at risk for cutaneous MCT, which accounts for up to 21% of all skin tumours (Thamm and Vail, 2007). The diagnosis of MCT by cytology or histopathology is straightforward in the majority of cases, but forming an accurate prognosis is more challenging (Welle et al., 2008). The biological behaviour of MCT is highly variable, in particular for intermediate-grade tumours. Some intermediate-grade tumours have a benign behaviour, while others exhibit aggressive growth and a high frequency of local and distant recurrence (Welle et al., 2008). Degradation of the ECM and basement membrane components and angiogenesis act in a coordinated manner in the growth and progression of several human and animal tumours (Loukopoulos et al., 2003; Patruno et al., 2009). These complex processes are mainly regulated by MMPs and their specific inhibitors (ECM and basement membrane degradation) and VEGF (angiogenesis). Few data are available on the roles of MMPs and VEGF-A in canine MCTs (Leibman et al., 2000; Loukopoulos et al., 2003; Patruno et al., 2009; Mederle et al., 2010). In veterinary oncology, this is the first study describing the expression of MMP-2, MMP-9, MT1-MMP, TIMP-2 and VEGF-A in canine MCTs using an integrated approach from mRNA to protein, by means of both qPCR and IHC. GZ was also performed to evaluate MMP-2 and MMP-9 gelatinolytic activity. MMP-2 and MMP-9 have been identified by GZ in 24 cases of canine MCT of histological grades 2 and 3 (Leibman et al., 2000) and in two cases of oral multifocal MCT by GZ and IHC (Loukopoulos et al., 2003). In those studies, normal tissue adjacent to the tumour was also considered. In the present study the activity of both latent and active forms of MMP-9 and MMP-2 was evaluated in grade 1, 2 and 3 MCTs and these data were compared with IHC and qPCR results. Both latent and active forms of MMP-9 and MMP-2 were found in almost all tumours and proenzyme activity was generally higher than the active form for both gelatinases. GZ showed a significant increase of the pro- and active forms of MMP-9 and MMP-2, correlated with histological grade, as previously observed by Leibman et al. (2000) in canine MCT and by Loukopoulos et al. (2003) in other tumour types. In general, the production of gelatinases is correlated with its biological behaviour: tumours produce more MMP-9 and MMP-2 than non-neoplastic tissue and malignant tumours produce significantly more MMP than their benign counterparts (Loukopoulos et al., 2003). The grade dependent expression observed through zymography was confirmed at the mRNA and protein levels. Increased MMP-9 mRNA expression was correlated with IHC score and with act-MMP-9 optical density by GZ. In contrast, MMP-2 expression was more variable: despite a grade-dependent increase in MMP-2 activity. Multifocal distribution of the immunohistochemical expression was observed and there

were no significant differences between grades in terms of percentage labelled cells or intensity of labelling. Moreover, there was high variability at the gene expression level for MMP-2. The results obtained here for MMP-2 are in contrast with previously published data. The invasion and malignant potential of many canine solid tumours (e.g. lymphoma, mammary tumours and oronasal tumours) have been associated with increased expression of MMP-2 (Hirayama et al., 2002; Papparella et al., 2002; Loukopoulos et al., 2003; Gentilini et al., 2005; Nakaichi et al., 2007; Aresu et al., 2011), while a minor role for MMP-9 in these tumours has been hypothesized because of its wide expression in normal cells, including macrophages and vascular smooth muscle cells (Nakaichi et al., 2007) or for the fact that the active form of MMP-9 was identified in only three cases among 51 tumours (Loukopoulos et al., 2003). In contrast, the results of the present study might suggest that MMP-9, rather than MMP-2, represents the key factor in ECM degradation by mast cells in canine MCTs and consequently in tumour aggressiveness and malignancy. MT1-MMP is one of the main activators of MMP-2. Mature active MT1-MMP is expressed on the cell surface, where it binds and activates pro-MMP-2 (Sato et al., 1994). Overexpression of MT1-MMP in tumour cells enhances human tumour growth and metastasis (Souliè et al., 2005). In veterinary oncology, MT1-MMP has been detected by IHC in canine mammary tumours, where its expression was observed in the cytoplasm of tumour and stromal cells (Papparella et al., 2002; Aresu et al., 2011). Both studies revealed increased expression of MT1-MMP in association with MMP-2 in canine mammary carcinomas and emphasised the contribution of stromal cells to the development of a pro-invasive micro-environment (Papparella et al., 2002; Aresu et al., 2011). In the present study, MT1-MMP mirrored MMP-2 behaviour at both the protein and mRNA levels. MMP-2 and MT1-MMP were both detected by IHC predominantly in the cytoplasm of tumour cells, and they showed multifocal distribution that increased in terms of percentage of labelled cells and intensity score with histological grade. At the gene expression level, MT1-MMP showed the same pattern of expression of MMP-2 and was positively correlated with MMP-2 expression. Thus, these results confirm the relationship between these two proteins in ECM degradation (Will et al., 1996). The gelatinolytic function of MMPs is also controlled by TIMPs. In particular, TIMP-2 has dual function of inhibition/activation of MMP-2 (Lambert et al., 2004). No reports are available of the role of TIMP-2 in canine MCT; this has been investigated only in canine mammary tumours (Kawai et al., 2006; Aresu et al., 2011). In the present study IHC and qPCR showed the lowest level of TIMP-2 expression in undifferentiated MCTs, which appears to be consistent with the primary MMP inhibitory role played by TIMPs

(Lambert et al., 2004). Imbalances in the activities of MMPs and TIMPs are involved in tumour progression (Liotta et al., 1991). MMP-2 mRNA increases with respect to TIMP-2 in more aggressive tumours (Onisto et al., 1995; Caenazzo et al., 1998; Nagel et al., 2004; Nakaichi et al., 2007); thus, the ratio of enzyme:inhibitor mRNA has been proposed as an early indicator of aggressiveness (Caenazzo et al., 1998; Nakaichi et al., 2007). In line with previous literature, a progressive increase in the MMP-2:TIMP-2 mRNA ratio according to tumour grade and malignancy was obtained here. To exert MMP-inhibiting or MMP-activating functions, TIMP-2 binds preferably to MMP-2, while TIMP-1 binds to MMP-9 (Stetler-Stevenson et al., 1989; DeClerck et al., 1991). Based on this evidence, the mRNA expression profile of TIMP-1 was examined here, but no statistically significant differences were obtained (data not shown). Nevertheless, the disturbed balance between MMPs and TIMPs in MCTs, as evidenced by the MMP-2:TIMP-2 mRNA ratio, was also confirmed by the MMP-9:TIMP-1 mRNA ratio, where in more aggressive MCTs (grade 3) an imbalance of about fourfold versus grade 1 was obtained. Thus, the MMP-2:TIMP-2 and MMP-9:TIMP-1 mRNA ratios might be useful for predicting the behaviour of MCTs. Angiogenesis is crucial for the development of solid tumours and it is known that tumour-associated vessels can supply oxygen and nutrients to tumour cells for several millimetres (Uchida et al., 2008). VEGF is a major regulator of angiogenesis and a potential autocrine growth factor for neoplastic cells (Kutet al., 2007). Recently, VEGF-A distribution has been evaluated in normal canine tissues (Uchida et al., 2008) and in canine mammary tumours, meningiomas, lymphomas and MCTs (Wolfesberger et al., 2007, 2008; Qiu et al., 2008; Matiasek et al., 2009; Millanta et al., 2010; Mederle et al., 2010). Several studies have demonstrated significant correlations between tumour grade and angiogenic factors and/or microvessel density in canine mammary gland tumours, basal cell tumours and squamous cell carcinomas (Maiolino et al., 2000; Restucci et al., 2002). Furthermore, intratumoural microvessel density has been evaluated in canine MCT and associated with tumour recurrence and mortality (Preziosi et al., 2004). For the first time, VEGF-A was measured in MCT samples by means of IHC and, by qPCR in the present study. Both methods confirmed that neoplastic mast cells constitutively expressed VEGF-A at both the mRNA and protein levels, as described by Rebuzzi et al. (2007). Additionally, increasing VEGF-A mRNA and protein expression according to histological grade was observed. A similar association was described by Patruno et al. (2009). The results obtained here might form the basis for development of new therapeutic strategies for canine MCT. For example, the use of an antiangiogenic compound in association with other chemotherapeutic agents might be beneficial in the management of non-resectable

grade 2 and 3 MCTs. As an example, tyrosine kinase inhibitors with both anti-angiogenic and direct anti-neoplastic activities (inhibition of c-KIT tyrosine kinase activity) might be employed. In this respect, surprisingly, transcriptional data obtained here for VEGF-A in grade 1, 2 and 3 MCTs were significantly correlated with c-KIT mRNA levels (data not shown). In conclusion, the results of the present study have shown the involvement of MMP-9 and VEGF-A in the progression and malignancy of canine MCT. These markers may be new novel therapeutic targets, but future studies should collect clinical outcome data to further understand the potential prognostic roles of these markers.

5. PHASE 3

5.1. SECTION 1: VEGF and MMP-9: biomarkers for canine lymphoma*

*Adapted with the permission of "Blackwell" from: Aresu L, Aricò A, Comazzi S, Gelain ME, Riondato F, Mortarino M, Morello E, Stefanello D, Castagnaro M. VEGF and MMP-9: biomarkers for canine lymphoma. Vet Comp Oncol. 2012 Apr 10. doi: 10.1111/j.1476-5829.2012.00328.x. © 2012 Blackwell Publishing Ltd. All rights reserved.

BACKGROUND

In human and veterinary oncology, one area of major promise is the identification of molecular markers, which may predict response to chemotherapy and tumour relapse, thereby offering predictive information. It has been reported that canine non-Hodgkin's lymphoma (cNHL) shows overlapping features with human non-Hodgkin's lymphoma (hNHL), allowing for the formulation of a comparative classification system and for the consideration of the dog as a possible spontaneous model for this tumour (Vail and MacEwen, 2000; Breen and Modiano, 2008). Cytokines play an important role in the pathogenesis of hNHL, and elevated plasma or tissue cytokine levels contribute to its progression (Pedersen et al, 2005; Labidi et al., 2009). Cytokines exert their effects on neoplastic and reactive cells, providing growth advantages for tumour cells in either an autocrine or a paracrine fashion (Sporn and Todaro, 1980). Angiogenesis plays a critical role in the initial development of cancer as well as in the metastatic spread. Previous studies have shown that angiogenesis is increased in canine lymphoma, by highlighting the higher micro-vessel density in neoplastic lymph nodes compared with the normal lymph nodes (Wolfesberger et al., 2008); however, the neovascularization was not correlated with VEGF immunoreactivity or overall survival time. Conversely, Gentilini et al. (2005) measured circulating VEGF, MMP-9 and MMP-2 in cNHL and found a longer disease-free interval in dogs with a low VEGF plasma level at admission; however, no differences in VEGF levels were noticed before and after treatment in eight dogs obtaining complete remission. When the included dogs were grouped according to some of the known prognostic factors, a significantly higher VEGF level was found in symptomatic dogs (substage b) compared with asymptomatic dogs (substage a). However, no attempt was made to evaluate the relationship between the level of angiogenic factors in cNHL and immunophenotype, cytological subtypes or grading. Moreover, activation of MMP-9 and MMP-2 was not evaluated.

AIM

By hypothesizing a relationship among cytokines and gelatinases, the aim of this work is the measurement of plasmatic pro-MMP-2, pro-MMP-9 and their activated forms, VEGF and TGF-*β* by gelatine zymography and an enzyme-linked *immunosorbent* assay, respectively, in a cohort of cNHL subdivided based on cytological classification and immunophenotype. Additionally, in a smaller group of dogs undergoing treatment, biomarkers levels at presentation will be correlated to clinical stage (to evaluate whether higher levels predicted a more advanced clinical stage), to

immunophenotype (B versus T) and to remission status at the end of chemotherapy (to evaluate whether variation in the angiogenic pathway may be related to remission status).

METHODS

Patients and samples

Plasma was obtained from dogs with lymphoma at various clinical stages, and from breed-, age and gender-matched healthy control dogs presenting for periodical examination. An informed consent was obtained from all owners according to the regulations of each institutional animal care committee. Dogs underwent complete staging work-up, including physical examination, complete blood cell count, flow cytometric and cytological analysis of nodal fine-needle aspirate, peripheral blood and bone marrow aspirate, thoracic radiography and abdominal ultrasound. Briefly, flow cytometric immunophenotype was determined as previously reported on fine needle aspiration of lymph nodes, peripheral blood and bone marrow samples (Gelain et al., 2008). The following monoclonal antibodies were used: CD45-PEb (clone YKIX716.13, Serotec, Oxford, UK), CD3-FITC (clone CA17.2A12, Serotec, T cells), CD4-FITC (clone YKIX302.9, Serotec, T-helper and neutrophils), CD8-PE (clone YCATE55.9, Serotec, T-cytotoxic/suppressor), CD5 (clone YKIX322.3, T-cell), CD21-PE (clone CA21D6 Serotec, mature B cells), CD34-PE (clone 1H6, Pharmingen, Becton Dickinson, San Jose, CA, USA, precursor cells), and CD79a (B-cells, clone HM57, Dako, Atlanta, GA, USA). Acquisition was performed with FACSCalibur (Becton Dickinson) and analysis was conducted by using a commercially available software (Cell Quest, Becton Dickinson). Lymphoma subtypes were classified based on the Kiel-updated cytological classification (Ponce et al., 2010). Plasma was processed by the Department of Veterinary Pathology, Hygiene and Health, University of Milan, and by the Department of Animal Pathology, University of Turin. At presentation, peripheral blood was sampled in sterile EDTA tubes, and the plasma obtained by centrifugation was put in separated polypropylene tubes and stored at −20°C. Each sample was centrifuged for 30 min at 1000 rpm before assaying. Owners of dogs with lymphoma were offered to treat their animals with multidrug chemotherapy, consisting of doxorubicin, vincristine, cyclophosphamide, L-asparaginase and prednisone (Simon et al., 2006). In these dogs, plasma was collected at three standard times (at diagnosis, halfway through the treatment and at the end of chemotherapy). The remission status of the treated dogs was recorded at each recheck examination.

MMPs analysis using gelatine zymography

MMP-2 and MMP-9 activity was studied by zymography, a technique revealing the gelatinase activity of latent pro-enzymes (zymogens) and mature MMPs. A 1:10 dilution was made from 10 µL of plasma into sample buffer, and 60 µL of the diluted sample was subjected to electrophoresis on an 8% SDS-PAGE gel co-polymerized with 0.1% gelatine. Other details about gelatine zymography are described in Phase 1.

Measurements of cytokine levels

Circulating VEGF and TGF-*β* levels were quantitatively analysed by an enzyme-linked immunosorbent assay (ELISA), using specific canine commercial kits (R&D Systems, Minneapolis, MN, USA) following manufacture instruction. Each sample was tested in duplicate. Spectrophotometer readings at 450nm (wavelength correction set to 570 nm) were performed using a Thermo Labsystems Multiskan Ascent Photometric plate reader (American Instrument Exchange, Haverhill, MA, USA). The lower limits of detection for VEGF and TGF-*β*1 are less than 19.5 and 4.61 pg/mL, respectively. Samples were prepared with HCl/NaOH/HEPES-activation solution, as recommended by the manufacturer (R&D Systems), to activate latent TGF-*β*1 into the immunoreactive form, the only one detectable by the kit.

Statistical analysis

The data were expressed as the mean±SEM. Comparison among cytokines and MMPs levels in dogs with different lymphoma subtypes and healthy controls was calculated by Student's *t*-test or analysis of variance (ANOVA). Differences in frequencies among all the cytokines were determined by $\chi 2$ analysis. Standard regression analysis, using Pearson and Spearman correlation coefficients, were used to determine the relationships between MMP, VEGF and TGF-*β*1 values. All tests were performed using NCSS 2000 software (Kaysville, UT, USA). Statistical significance was set at $p<0.05$.

RESULTS

Thirty-seven dogs with lymphoma and 10 healthy controls were evaluated. Among the lymphoma cases, 21 were of B-cell immunophenotype (19 high-grade, 1 low-grade and in 1 case no lymph node glass smear was available) and 16 of T-cell immunophenotype (9 high-grade and 7 low grade). The main B-cell subtype was centroblastic polymorphic (17 of 21), whereas in T-cell

lymphoma (including high- and low-grade) no predominant cytological subtype was detected. Thirty-four dogs underwent complete staging work-up: 20 lymphomas were of stages III–IV, whereas 14 of stage V. Twenty-nine of the 37 dogs were treated and received the same CHOP-based chemotherapeutic protocol (Gelain et al., 2008). At the end of the 12 weeks of treatment, 12 (41.4%) dogs were in complete remission, 3 (10.3%) obtained partial remission, 3 (10.3%) dogs had stable disease and 11 (38.0%) dogs experienced progressive disease. When considering immunophenotype and outcome, among the 18 treated dogs with B-cell lymphoma, 9 obtained complete remission, 2 partial remission, 2 stable disease and 5 experienced progressive disease. Among the 11 treated dogs with T-cell lymphoma, three obtained complete remission, one partial remission, one stable disease and six experienced progressive disease.

Correlation between MMPs and cytokines profiles at admission and disease status, clinical stage and immunophenotype.

The quantification of gelatinases through gel zymography showed similar values of pro-MMP-9 in tumour and control dogs (B-cell lymphomas = 93.1 ± 6.4 a.u.; T-cell lymphomas = 97.9 ± 9.4 a.u. and control dogs = 92.3 ± 2.5 a.u.). However, dogs with lymphoma showed a significantly higher catalytic activity of MMP-9 ($p<0.01$) compared with healthy controls (91.7 ± 4.8 a.u. versus 12.4 ± 2.1 a.u., respectively; Table 1). When considering the cytokine levels and clinical stage, statistically significant higher expression of act-MMP-9 was found in stage V B-cell lymphomas (95.3 ± 3.4 a.u.) than in stage III/IV B-cell lymphomas (83.4 ± 4.7 a.u.; $p<0.01$). Higher expression of act-MMP-9 was found in stage V T-cell lymphomas (113.7 ± 3.2 a.u.) than in stage III/IV T-cell lymphomas (94.7 ± 2.1 a.u.; $p<0.05$). When examining the immunophenotype, T-cell lymphomas presented a higher concentration of act-MMP-9 at the time of diagnosis (103.3 ± 2.9 a.u.) than B-cell lymphomas (89.2 ± 8.8 a.u.), and this was statistically significant ($p<0.05$). Neither lymphoma nor control dogs showed expression of act-MMP-2. Plasma levels of pro-MMP-2 were not significantly increased in lymphoma dogs (104.3 ± 10.2 a.u.) with respect to control dogs (98.2 ± 3.9 a.u.; Fig. 1; Table 1). Plasma VEGF levels in dogs with lymphoma were higher than that in controls (59.3 ± 14.3 pg/mL versus 40.1 ± 10.4 pg/mL, respectively; $P<0.05$), and this was statistically significant ($P<0.05$; Table 1). Also, VEGF plasmatic levels were significantly higher ($p<0.01$) in stage V B-cell lymphomas (58.3 ± 4.5 pg/mL) than in stage III/IV B-cell lymphomas (49.1 ± 2.1 pg/mL), and in stage V T-cell lymphomas (78.1 ± 3.7 pg/mL) than in stage III/IV T-cell lymphomas (63.1 ± 4.5 pg/mL). In dogs with B-cell lymphoma, the mean VEGF value at diagnosis was lower when compared with dogs

with T-cell lymphoma (55.4 ± 21.3 pg/mL versus 67.4 ± 19.1 pg/mL, respectively). A positive correlation between concentrations of act-MMP-9 and VEGF plasma levels was found in all lymphoma dogs (r = 0.78, p<0.001). Concerning plasma TGF-*β* levels, the median values were similar between B- and T-cell lymphoma (11377.6 pg/mL and 10343.4 pg/mL, respectively). Although not statistically significant, the mean plasma TGF-*β* value in control healthy dogs (19185.5 pg/mL) was higher when compared with lymphoma dogs (Table 1). At the time of diagnosis, VEGF levels were significantly lower in low-grade (59.7 ± 3.3) compared with high-grade (76.4 ± 4.8) T-cell lymphomas (p<0.05). No such differences were observed for MMP-2, MMP-9 and TGF-*β*.

Correlation between MMPs analysis and cytokines profiles at admission and remission status at the end of chemotherapy.

Plasma was obtained at admission in 29 dogs undergoing the same chemotherapeutic protocol. MMP and cytokines profiles did not differ between dogs obtaining remission (complete and partial) or experiencing progressive disease, thereby not being useful in predicting treatment response.

MMPs analysis and cytokines profiles during chemotherapy and follow-up.

Plasma was serially obtained from 13 dogs (10 B-cell and 3 T-cell lymphomas) undergoing chemotherapy. In these dogs, pro-MMP-9, pro-MMP-2 and act-MMP-2 levels did not change at the three standard times. However, act-MMP-9 was significantly decreased in all B-cell lymphoma dogs at the end of chemotherapy (38.1 ± 12.3 a.u.; p<0.01; Fig. 3). In contrast, no evident modifications of the quantitative activity of act-MMP-9 were observed in the T-cell lymphoma dogs (101.3 ± 10.0 a.u.; Fig. 2 and Fig. 3). In dogs undergoing chemotherapy, 8 of 9 cases of B-cell lymphoma showed decreased VEGF values at the end of treatment in the order of 19.47 pg/mL. The mean value of the reduction, excluding one case of anaplastic B-cell lymphoma that presented a rise of VEGF levels, was 20.7 pg/mL (p<0.05). In the three dogs with T-cell lymphoma undergoing treatment, the mean value of the rise of plasma VEGF was 15.6 pg/mL at the end of treatment.

Table 1. Baseline values of MMP-9 (pro and act), VEGF and TGF-*β* in controls, B-cell lymphomas and T-cell lymphomas (expressed as the mean ±SEM)

	CONTROLS	**LYMPHOMA B**	**LYMPHOMA T**
Pro-MMP9 (a.u.)	92.3 ± 2.5	93.1 ± 6.4	97.9 ± 9.4
Act-MMP9 (a.u.)	12.4 ± 2.1	89.2 ± 8.8**	103.3 ± 2.9**
VEGF (pg/ml)	40.1 ± 10.4	55.4 ± 21.4*	67.4 ± 19.1*
TGF- β (pg/ml)	19185.5 ± 7843.4	11377.6 ± 3412.7	10343.4 ± 5430.9

*$p<0.05$. **$p<0.01$ versus controls.

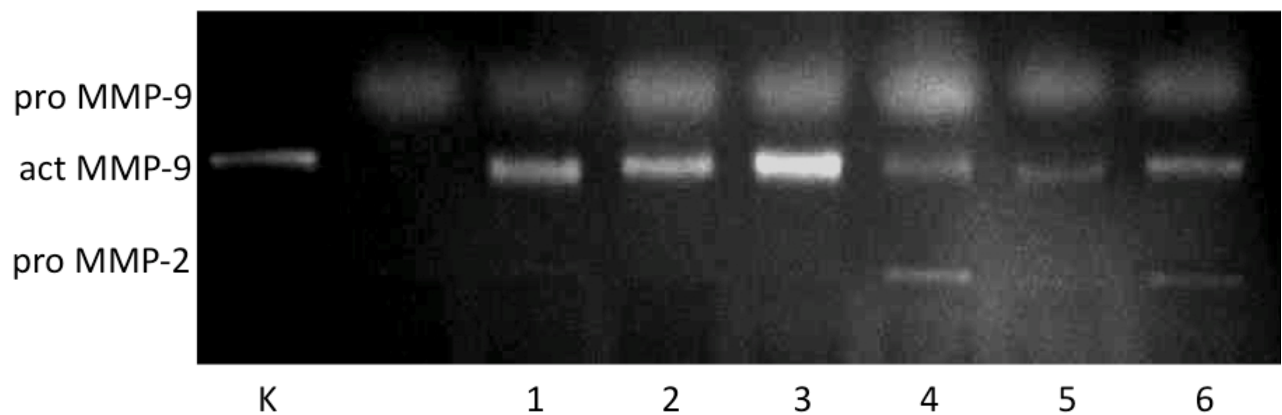

Fig. 1. Representative MMP zymography from seven samples. Lanes 1, 2 and 3 are T-cell lymphomas. Lanes 4, 5 and 6 are B-cell lymphomas.

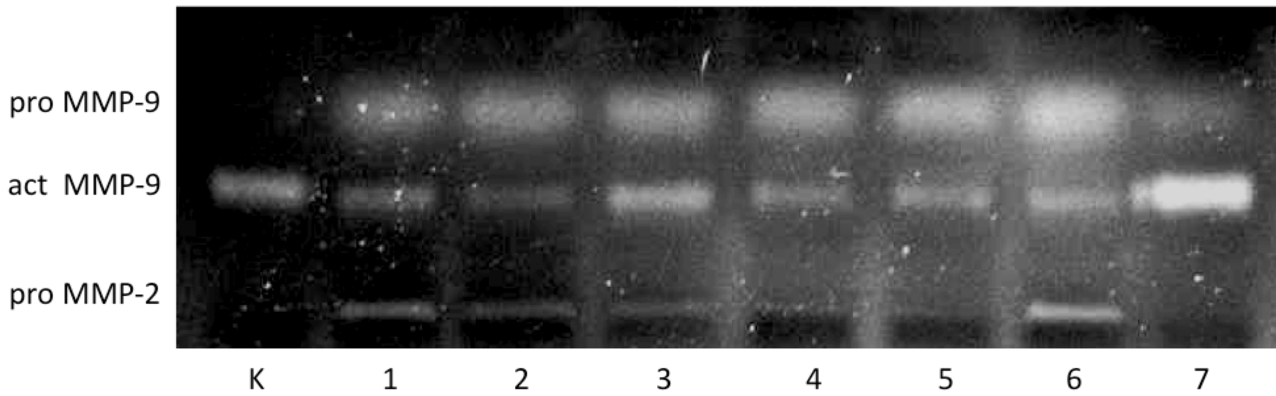

Fig. 2. Representative MMP zymography from three dogs during chemotherapy. Lanes 1, 2 and 3: dog 6, B-cell lymphoma, respectively, at time 2, time 1 and time 0. Lanes 4, 5 and 6: dog 17, B-cell lymphoma, respectively, at time 2, time 1 and time 0 in. Lane 7: T-cell lymphoma in dog 35.

LYMPHOMA T
LYMPHOMA B
END OF TREATMENT
HALF TREATMENT
TIME 0
0 20 40 60 80 100 120
Act MMP-9 concentrations (a.u.)

Fig. 3. Plasma concentrations of act-MMP-9 (mean values) in B- and T-cell lymphomas at the three standard times during the chemotherapy protocol.

DISCUSSION

Cytokines act as important key regulators of the tumour microenvironment, by influencing survival and proliferation of neoplastic and vascular cells. In people with NHL, angiogenic factors are emerging as a powerful prognostic tool (Baly et al., 1993; Pedersen et al, 2005; Labidi et al., 2009). In literature, few studies are available in veterinary oncology on the role of VEGF and MMPs (Hirayama et al., 2002; Gentilini et al., 2005; Yonemaru et al., 2006; Zizzo et al., 2010; Aresu et al., 2011) and the clinical impact of early and serial monitoring of these cytokines has not been extensively studied. Here, possible plasma biomarkers were identified having predictive relevance in canine lymphoma. Specifically, the results of present study show that VEGF and act-MMP-9 levels were significantly higher (1) in dogs with lymphoma when compared with controls, (2) in T-cell lymphomas compared with B-cell lymphoma at admission and that (3) VEGF was higher in high-grade compared with low grade T-cell lymphoma. MMP-9 and VEGF are two of the most potent factors involved in angiogenesis. It is well known that, in addition to its role in proteolytic degradation, MMP-9 can also release angiogenic factors that bind to ECM, such as VEGF. MMP-9 is also a functional component of the angiogenic switch during multistage carcinogenesis, as it increases the availability of angiogenesis inducers (Bergers et al., 2000; Moehler et al., 2003). In this study, a correlation between the levels of VEGF and act-MMP-9 and lymphoma immunophenotype was found; the explanation of which could rely on the more aggressive biological behaviour and rapid spread that characterize T-cell lymphoma. Indeed, it has been documented that canine T-cell lymphoma usually harbours a poor prognosis (Ponce et al., 2004): in the early stage, high-grade nodal lymphoma does not destroy tissue boundaries, thereby not showing an invasive growth pattern. However, as the tumour progresses, it may become locally invasive and tends to disseminate, as partially shown by predictive correlations exhibited in the present study between plasma levels of VEGF and MMP-9. However, it is a matter of fact that canine lymphoma encompasses a wide range of distinct entities showing different biologic behaviour; as a consequence, not all T-cell lymphomas carry the same prognosis (Ponce et al., 2010). For this reason it was decided to group the cases based on their morphological aspect as well, and, interestingly, the low-grade T-cell lymphomas had a significant lower level of VEGF, being in accordance with results obtained in human medicine (Salven et al., 1997). On the basis of these findings it may be hypothesized that a different angiogenic pathway occurs in low- and high-grade T-cell lymphomas. Because B-cell lymphomas were mainly of high-grade in the series considered in this study, it was not possible to discriminate the VEGF data. In hNHL, VEGF and

MMP-9 expressions correlate with: (1) subtype, (2) grade, (3) clinical course and (4) survival (Salven et al., 1997; Sakata et al., 2004). Patients with elevated VEGF and MMP-9 levels have a higher likelihood of recurrence or death than patients with low-angiogenic NHL. The role of VEGF and act-MMP9 in different stages of lymphoma (III–IV versus V) was analysed. Their levels were found to be different between lower and higher stages, thereby suggesting a possible role of cytokine levels to differentiate between advanced and early cancer. According to the results of the present, cytokines levels at presentation did not correlate to remission status obtained at the end of chemotherapy. The reason behind this finding may reside in the low number of treated cases or in distinct characteristics of the dog groups studied. It may be possible that group stratification according to clinical stage and immunophenotype will eventually lead to outcome correlation. Intriguingly, by serially determining cytokines level during and after chemotherapy, plasma VEGF and MMP-9 was recognized as a dynamic follow-up parameter in B-cell lymphoma. Indeed, both biomarkers decreased significantly from admission to the end of treatment in these dogs, thereby being in agreement with several human studies indicating that VEGF and MMP-9 might predict treatment response (Sakata et al., 2004; Pedersen et al, 2005; Labidi et al., 2009). At midterm plasma check, the biomarkers levels were not significantly different when compared with the levels obtained at admission (data not shown). As a consequence, it may be assumed that the most relevant changes in the production of these markers are reflected at the end of treatment, being attributable to the persistence of high circulating levels of VEGF and MMP-9 during chemotherapy. Conversely, in T-cell lymphomas, VEGF levels increased at the end of the treatment, whereas MMP-9 showed no changes. More data are needed to evaluate the plasma kinetics of these two biomarkers in dogs with lymphoma to predict a possible correlation between their synthesis and the responses to chemotherapy. On the other hand, the role of MMP-2 seems to be irrelevant in the lymphoma microenvironment. Pro-MMP-2 levels in dogs with lymphoma were not significantly increased, regardless of the immunophenotype. TGF-*β* showed lower plasma levels in lymphoma dogs compared with controls; however, there was a high individual variability at diagnosis. Furthermore, no correlation between plasma TGF-*β* levels and lymphoma immunophenotype was found, and there was no correlation at the three standard treatment times. It may be hypothesized that a reduction of TGF-*β* reflects the ability of the tumour cells to acquire resistance to the anti-proliferative signals of TGF-*β*. In NHL and other haematological malignancies, the aberrant expressions of receptors (types I, II and III) and mutations in TGF-*β* signalling cascade have been described, demonstrating that cancer cells frequently acquire

resistance to the anti-proliferative signals of TGF-*β* (Dong and Blode, 2006). The same may hold true for dogs with lymphoma; however, additional studies with larger patient numbers are warranted to verify these observations. In conclusion, VEGF and act-MMP-9 levels were significantly higher in dogs with lymphoma compared with healthy controls. Furthermore, VEGF and act-MMP-9 levels were higher in T-cell lymphomas and in dogs with a more advanced disease, thereby providing a new tool that might help oncologists in predicting outcome. Future studies need to be conducted to highlight the potential role of anti-angiogenetic agents in the treatment of different subtypes of canine lymphoma.

5.2. SECTION 2: Matrix metalloproteinases and vascular endothelial growth factor expression in canine lymphoma[§]

from: Aricò A, Giantin M, Gelain M, Riondato F, Mortarino M, Comazzi S, Dacasto M, Castagnaro M, Aresu L. Submitted

[§] Presented at the 2012 World Veterinary Cancer Congress, Paris, France, March 1-2-3, 2012.

AIM

The aim of the second study on the canine lymphoma was to assess the gene expression profiles of MMP-2, MMP-9, MT1-MMP, TIMP-1, TIMP-2, RECK and VEGF-A and the protein levels of MMP-2, MMP-9 and VEGF-A in canine B and T-cell lymphoma.

METHODS

Caseload and classification

Fine-needle aspirates (FNAs) of enlarged lymph nodes obtained from dogs with lymphoma were collected. Samples were sent by the referring veterinarians to the Department of Veterinary Pathology, Hygiene and Health at the University of Milan, and to the Department of Animal Pathology at the University of Turin for diagnostic purposes. The different subtypes were described according to the updated Kiel classification (Fournel-Fleury et al., 1997) by considering pleomorphism; cell size; nuclear shape; chromatin density; mitotic index; the number, size and distribution of nucleoli; and the extension and basophilia of the cytoplasm. The immunophenotype was determined by means of flow cytometry with the use of the following monoclonal antibodies: CD45-PEb (clone YKIX716.13, Serotec, Oxford, UK, leukocytes), CD3-FITC (clone CA17.2A12, Serotec, T cells), CD4-FITC (clone YKIX302.9, Serotec, T-helper cells and neutrophils), CD8-PE (clone YCATE55.9, Serotec, T-cytotoxic/suppressors), CD5 (clone YKIX322.3, T-cells), CD21-PE (clone CA21D6 Serotec, mature B cells), CD34-PE (clone 1H6, Pharmingen, Becton Dickinson, San Jose, CA, precursor cells), and CD79a (clone HM57, Dako, Atlanta, GA, all stages of B-cells). Data acquisition was performed by using a FACSCalibur (Becton Dickinson), and the analysis was conducted with a commercially available software (Cell Quest, Becton Dickinson). The expression of specific lineage markers defined the lineages of lymphoma: CD3, CD5, CD4, and/or CD8 for T-cell lymphoma and CD21 and/or CD79a for B-cell lymphoma. A positive staining referred to the antigen expression in at least 20% of the gated cells. Samples being characterized by an ambiguous diagnosis, low cellularity or viability were excluded from the present study. Five healthy dogs matched according to age, breed, and gender with no relevant peripheral lymph node alterations served as controls. Informed consent was obtained from all owners according to the regulations of each institutional animal care committee.

Sampling procedure

Two samples were obtained from each lymph node FNA for routine cytological exam and flow cytometry, and for immunocytochemical analysis and total RNA extraction. For flow cytometry, cells were suspended in 0.5 mL of RPMI 1640 medium (Sigma Aldrich, Munich, Germany) containing 5% foetal bovine serum (Sigma Aldrich) and 0.2% sodium azide (Sigma Aldrich) at room temperature. The remaining material was washed twice in the same medium, re-suspended in RNAlater® solution (Life Technologies, Foster City, CA) and stored at −20°C for total RNA isolation. For total RNA isolation, at least 0.5 mL of each cell suspension containing of 2×10^6 cell/mL of good viability was required.

Quantitative real-time RT-PCR (qRT-PCR)

The total RNA was isolated using the RNeasy Mini Kit (Qiagen, Milan, Italy); according to the manufacturer's instructions. RNA was isolated from both cell pellets and RNAlater® suspensions, as recommended by Dunmire et al. (2002). To avoid genomic DNA contamination, on-column DNase digestion with the RNase-Free DNase set was performed. cDNA synthesis, primers and the formulation of final value are reported in Phase 1. Primer pairs and human UPL probes for MMP-2, MMP-9, MT1-MMP, TIMP-1, TIMP-2, RECK and VEGF-A amplification were described previously (Aresu et al. 2011; Giantin et al. 2012). In the present study, VEGF-164, the VEGF-A splice variant markedly expressed in dogs and highly conserved among species (Usui et al., 2004) was also considered. Canine VEGF-164 was amplified using the primer pair 5′-CGT GCC CAC TGA GGA GTT-3′ (forward) and 5′-AAG GCC CAC AGG GAT TTT CT-3′ (reverse) and human UPL probe #9. Calibration curves using a 4-fold serial dilution of a cDNA pool revealed PCR efficiencies near two and error values < 0.2 (see Table 1).

Table 1. qRT-PCR assay parameters: primer concentration, efficiency, linearity and dynamic range.

Genes	Primer concentration (nM)	Efficiency	Error	Dynamic range (Cp)
CGI-119	F600/R300	1.902	0.00291	23.90 – 32.39
MMP-2	F600/R600	2.002	0.03450	25.57 – 32.68
MMP-9	F300/R300	1.998	0.05740	30.50 – 36.28
MT1-MMP	F600/R600	2.001	0.02890	24.64 – 31.68
TIMP-1	F600/R300	2.000	0.03110	25.24 – 31.25
TIMP-2	F300/R300	2.034	0.02890	24.50 – 31.38
RECK	F600/R300	2.010	0.06310	27.05 – 33.78
VEGF-A	F300/R300	2.028	0.01020	26.56 – 32.49
VEGF-164	F300/R300	1.998	0.02690	28.54 – 35.19

F, forward primer; R, reverse primer

Immunocytochemical analysis

The protein expression levels of MMP-2, MMP-9 and VEGF-A were evaluated by immunocytochemistry. The cellular suspension designated for immunocytochemical analysis was prepared by cytospin and fixed with acetone and methanol (Sigma Aldrich). After fixation, the primary antibody incubation step was performed by an automated system for all antibodies (Ventana Medical Systems). The antibodies used in this study were the following: anti-human MMP-9, Clone C-TERM (1:200; Millipore S.p.A, Milan, Italy); anti-human MMP-2, Clone Ab-7 (1:100; Thermo Fisher Scientific Inc., Kalamazoo, Michigan, USA); and anti-human VEGF-A, Clone A-20 – sc:152 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA). The details of the immunohistochemal performance and the labelling valuation are described in Phase 1.

Statistical analysis

The statistical analysis of the gene expression results was performed using Mann–Whitney tests, whereas the immunocytochemistry data were analysed using the Kruskal-Wallis test followed by Dunn's post-test. A non-parametric Spearman correlation analysis was used to determine the potential relationship between target genes. GraphPad Prism 5 software (San Diego, California, USA) was used for all statistical evaluations. Statistical significance was set at $p<0.05$. Finally, Grubbs' test was used to identify potential outliers.

RESULTS

Clinical results

Forty-seven dogs with lymphoma were enrolled. Based on cytological and flow cytometric evaluation, there were 26 B-cell lymphomas (22 high-grade, HG, and 4 low-grade, LG), and 21 T-cell lymphomas (13 HG and 8 LG).

qRT-PCR

The gene expression results for healthy control lymph nodes, B-cell and T-cell lymphomas are summarised in Table 2. MMP-2 and TIMP-2 mRNA levels in the healthy control lymph nodes were significantly higher than in lymphomas. A significantly higher MMP-9 mRNA expression was observed in T-cell lymphomas compared to B-cell lymphomas and healthy controls. TIMP-1 exhibited similar results to MMP-9: in particular, T-cell lymphomas exhibited significantly higher TIMP-1 mRNA expression than B-cell lymphomas and healthy controls. MT1-MMP displayed the opposite expression pattern of MMP-2 in T-cell lymphomas. Furthermore, significantly higher MT1-MMP expression was observed in T-cell lymphomas compared to B-cell lymphomas. MT1-MMP mRNA levels were significantly higher in control lymph nodes than in B-cell lymphomas. When considering the tissue inhibitor RECK, statistically significant differences were observed between B-cell and T-cell lymphomas, and between T-cell lymphomas and healthy controls. B-cell and T-cell lymphomas had similar VEGF-A and VEGF-164 expression profile, moreover T-cell lymphomas showed significantly higher expression of VEGF-A with respect to controls. Interestingly, a higher MMP-9, MMP-2, MT1-MMP, VEGF-A, VEGF-164 and TIMP-1 mRNA expression was observed in HG T-cell lymphomas compared to LG T-cell lymphomas, although the differences were not statistically significant (Table 3). The same analysis was not performed in B-cell lymphomas because of the low number of LG cases included in the study. The expression of

MMP-9, MT1-MMP, TIMP-1 and RECK was higher in HG T-cell lymphomas compared to HG B-cell lymphomas (*p*<0.05). Although not statistically significant, VEGF mRNA expression showed the same trend. Significant correlations between MMP-2 and TIMP-2 (*p*<0.001; Spearman r=0.82) and between MMP-9 and VEGF-A (*p*<0.01, Spearman r=0.67) were found in T-cell lymphomas.

Table 2. MMP-9, MMP-2, MT1-MMP, TIMP-1, TIMP-2, VEGF-A, VEGF-164 and RECK mRNA expression in control lymph nodes, B-cell and T-cell lymphomas.

Genes	Control Lymph Nodes	B-cell Lymphoma	T-cell Lymphoma
MMP-2	0.96 ± 0.23[a,b]	0.24 ± 0.28	0.16 ± 0.29
TIMP-2	0.85 ± 0.34[a,b]	0.15 ± 0.20	0.16 ± 0.29
MT1-MMP	0.61 ± 0.17[a,b]	0.11 ± 0.11[c]	0.94 ± 0.99
MMP-9	0.13 ± 0.11[b]	0.12 ± 0.04[c]	0.69 ± 0.11
TIMP-1	0.15 ± 0.09[b]	0.16 ± 0.22[c]	0.66 ± 0.75
RECK	0.29 ± 0.12[b]	0.06 ± 0.12[c]	0.87 ± 1.12
VEGF-A	0.37 ± 0.19[b]	0.71 ± 0.98	0.89 ± 1.37
VEGF-164	0.57 ± 0.23	0.63 ± 0.92	0.90 ± 1.38

Data are expressed as the mean ± standard error.

[a, b, c] Significant differences between control lymph nodes and B-cell lymphoma, control lymph nodes and T-cell lymphoma, B-cell lymphoma and T-cell lymphoma, respectively (Kruskal-Wallis test followed by Dunn's post test; c: $p < 0.05$).

Table 3. MMP-9, MMP-2, MT1-MMP, TIMP-1, TIMP-2, VEGF-A, VEGF-164 and RECK mRNA expression in HG and LG T-cell lymphomas.

Genes	**High grade**	**Low grade**
MMP-2	0.18 ± 0.22	0.17 ± 0.20
MMP-9	0.82 ± 0.15	0.12 ± 0.28
MT1-MMP	1.01 ± 1.08	0.81 ± 0.86
TIMP-1	1.07 ± 1.98	0.63 ± 0.92
TIMP-2	0.15 ± 0.28	0.16 ± 0.32
RECK	0.93 ± 1.26	1.67 ± 2.76
VEGF-A	1.21 ± 1.65	0.35 ± 0.43
VEGF-164	1.11 ± 0.92	0.39 ± 0.88

Data are expressed as relative quantification values (mean ± SD)

Immunocytochemical analysis

Immunocytochemical results are summarised in Table 4. The protein expression levels of MMP-9, MMP-2 and VEGF-A were significantly higher in T-cell lymphomas compared to B-cell lymphomas (Fig. 1, 2). A statistically significant difference between the healthy control FNAs and the lymphoma group (p<0.05) was also observed. Moreover, MMP-9 and VEGF protein expression was significantly correlated in B-cell lymphomas (p<0.0002; r=0.72) and T-cell lymphomas (p<0.006; r=0.69).

Table 4. MMP-9, MMP-2 and VEGF-A protein expression in B-cell lymphoma and T-cell lymphoma.

Target proteins	**Healthy control FNAs**	**B-cell Lymphoma**	**T-cell Lymphoma**	***Significance***
MMP-9	0.57 ± 0.53	19.91 ± 28.99	68.13 ± 83.22	*P<0.05*
MMP-2	0.14 ± 0.37	14.45 ± 22.50	15.64 ± 21.96	*P<0.05*
VEGF-A	0.14 ± 0.37	6.4 ± 13.71	12.43 ± 17.88	*P<0.05*

Data are expressed as immunostaining scores (mean ± SD)

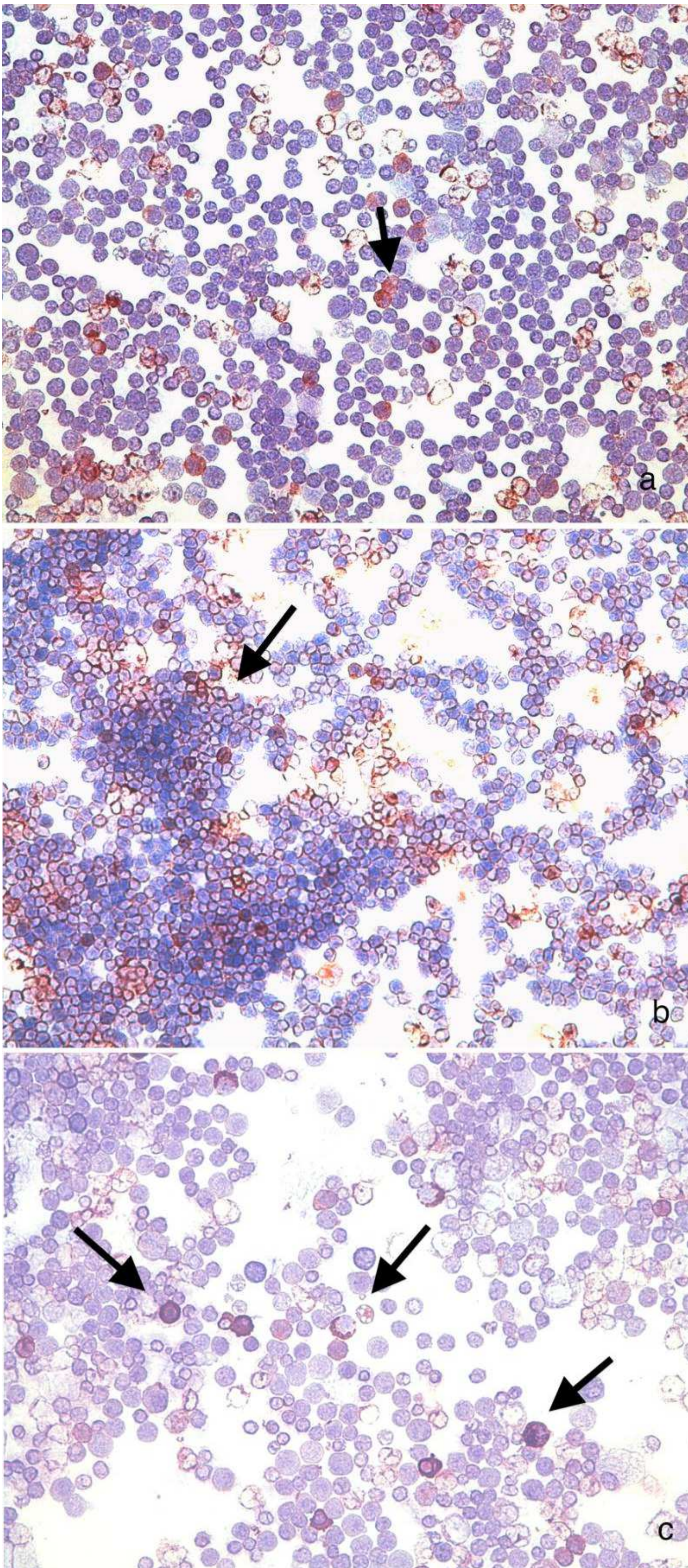

Fig. 1. B-cell lymphoma. FNAs. (a) low number of positive immunoassayed lymphoid tumour cells for MMP-2 antibody (arrow); (b) numerous positive immunoassayed lymphoid tumour cells for MMP-9 antibody (arrows); (c) plasma cells and low percentage of immunoassayed lymphoid tumour cells for VEGF antibody (arrow). Immunocytochemistry, 400X.

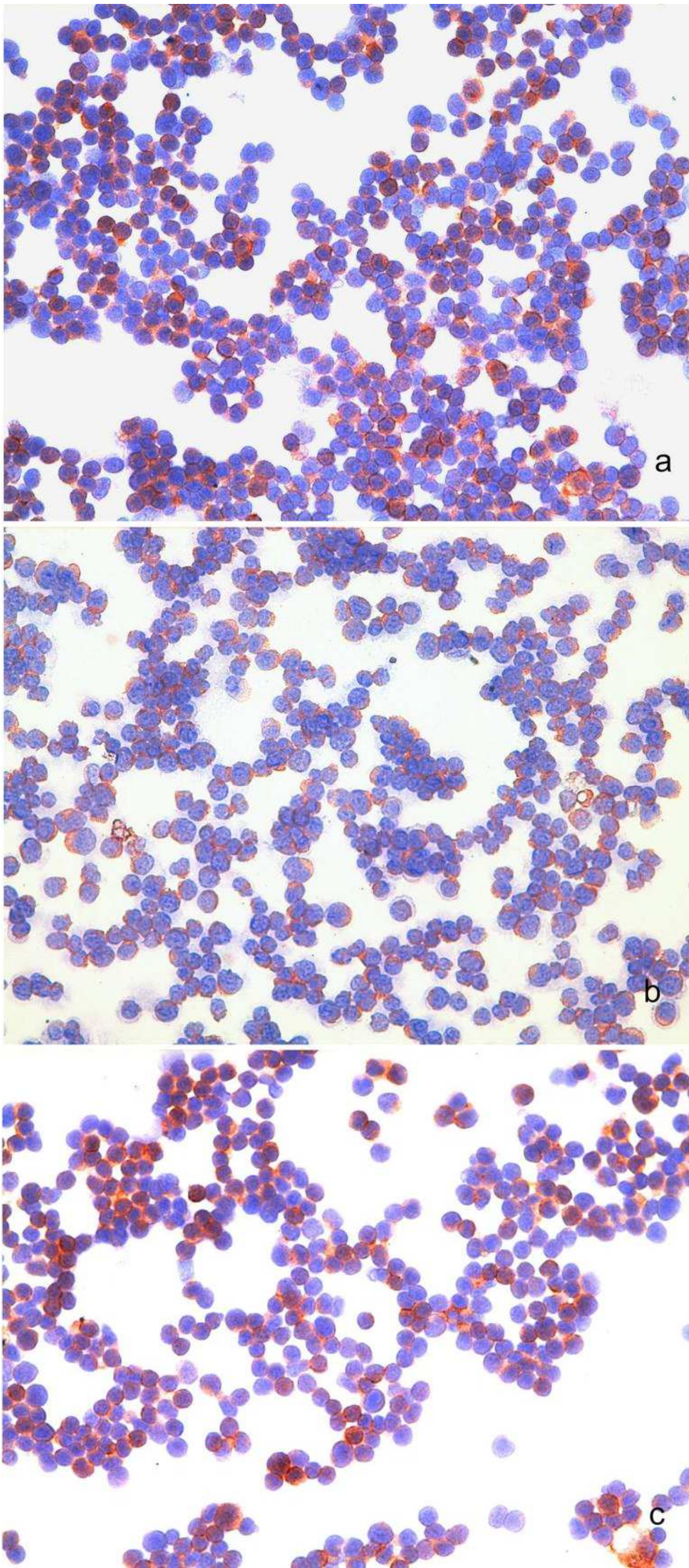

Fig. 2. T-cell lymphoma. FNAs. intense and widespread positive immunoassayed lymphoid tumour cells for (a) MMP-2 antibody, (b) MMP-9 antibody and (c) VEGF antibody. Immunocytochemistry, 400X.

DISCUSSION

In human haematologic malignancies, VEGF and different enzymes involved in ECM remodelling, including MMPs, are considered molecules for early diagnosis and prognostic assessment (Hayashibara et al., 2002; Pedersen et al., 2005; Buggins et al., 2011). The activation and inactivation of MMPs are regulated by TIMPs, which play a role in regulation of cell growth, and neovascularisation (Egeblad et al. 2002). In this study the expression of different MMPs, their regulators and VEGF, assessing mRNA and protein expression profiles, were analysed in canine lymphoma. Significantly higher MMP-9 mRNA and protein expression levels were observed in T-cell lymphomas compared to B-cell lymphomas and healthy control lymph nodes, indicating that MMP-9 expression may be associated with tumour phenotype. Moreover, the difference was significantly higher in HG T-cell lymphomas compared with HG B-cell lymphomas. Biologically, T-cells migrate across ECM barriers during the inflammatory process towards target tissues and the activation of MMP-9 causes the alteration of adjacent connective tissues and the degradation of collagen type IV. This result indicates that during tumour growth, neoplastic T-cells may increase the production of MMP-9, aimed at invading blood and other organs (Moehler et al., 2003). This data was confirmed by immunohistochemical results where T-cells expressed high amount of MMP-9 protein. Moreover, the gene and protein expression results for MMP-9 seem to confirm the prominent biologic behaviour of HG T-cell lymphoma characterized by invasion and metastatic spread, rendering this malignancy highly aggressive (Marconato et al., 2012). The highest levels of TIMP-1 were observed in T-cell lymphomas compared to B-cell lymphomas and controls. The highest levels were associated with HG T-cell lymphomas. This result supports the hypothesis that MMP-9 and TIMP-1 may act in concert in canine T-cell lymphoma. MMP-9 is frequently expressed and secreted with TIMP-1 by canine neoplastic cells (Aresu et al., 2011). These molecules are associated with a more aggressive clinical behaviour in human lymphomas, and they appear to exert their influence through two different mechanisms: MMP-9 causes ECM degradation, whereas TIMP-1 has an anti-apoptotic action (Kossakowska et al., 1991, and 1999). Furthermore, in human, increased TIMP-1 expression was observed in HG non-Hodgkin’s lymphomas and in advanced stage disease, leading the investigators to postulate that TIMP-1 may have lymphoid growth factor activity (Kossakowska et al., 1991). MMP-2 results were comparable to TIMP-2. Interestingly, in T-cell lymphomas, qRT-PCR analysis for MMP-2 revealed significant positive correlations with TIMP-2 and a negative correlation with MT1-MMP. By immunohistochemistry, neoplastic lymphocytes exhibited positive immunoreaction to MMP-2, confirming the protein

expression of this enzyme by neoplastic cells. Indeed, the MMP-2 antibody used in this work allows identifying both the inactive and active form of the enzyme. Gelatine zymography of T and B neoplastic cells was performed to investigate the activity of MMP-2 and MMP-9, but it was unable to detect any catalytic process. This discrepancy may be explained by the characteristics of the methodologies: immunocytochemistry shows the distribution and localisation of specific antigens within the cells, qRT-PCR can detect small quantities of transcripts in a relatively low number of cells, whereas gelatine zymography is sensitive to the number of cells preventing detection (Snoek-van Beurden and Von Der Hoff, 2005). Interestingly, higher MT1-MMP mRNA expression levels were observed in T-cell lymphomas compared to B-cell lymphomas and in HG T-cell lymphomas with respect to the LG counterparts. This result supports the important role of MT1-MMP in tumour invasion and also to promote tumour angiogenesis, by degrading the fibrin matrix that surrounds newly formed vessels (Egeblad et al., 2002). In both T- and B-cell lymphomas, VEGF expression at the transcript and protein level was observed. Tumour vascularisation is higher in lymphomas than in control lymph nodes and increases in HG T-cell lymphomas. VEGF is also present in lymphoma cells. Moreover, the mRNA and protein VEGF results were correlated with MMP-9 results in T-cell lymphomas. These data appear to be in accordance with the previous work of this project, where a close relationship between MMP-9 and VEGF plasmatic levels in canine lymphomas was reported (Aresu et al., 2012). The same results were observed in canine mast cell tumours, where release of VEGF by mast cells is correlated with higher MMP-9 production (Giantin et al., 2012). Indeed, the feedback regulation between MMP-9 and VEGF is assumed to be implicated in the angiogenic switch. In conclusion, the present data provides new information in the complex interaction of the migration/adhesion genes and canine lymphoma. Further efforts should be directed towards clarifying the detailed molecular mechanisms, including signal transduction and polymorphisms, which may lead to novel therapeutic strategies. The tumour vasculature is an attractive target for lymphoma therapy. The results from this study also indicate that differences between lymphoma subtypes must be taken into account in the selection of the most suitable dogs for trials with anti-angiogenic agents.

5.3. SECTION 3: Matrix metalloproteinases and vascular endothelial growth factor expression in canine leukaemias*§

*Adapted with the permission of "Elsevier" from: Aricò A, Giantin M, Gelain M, Riondato F, Mortarino M, Comazzi S, Dacasto M, Castagnaro M, Aresu L. Vet J. 2012 Nov 7. pii: S1090-0233(12)00426-1. doi: 10.1016/j.tvjl.2012.10.004. © 2012 Elsevier Ltd. All rights reserved.

§ Presented at the 2012 World Veterinary Cancer Congress, Paris, France, March 1-2-3, 2012.

BACKGROUND

Acute lymphoblastic leukaemia (ALL) is an uncommon but devastating disease in dogs; it is frequently and rapidly fatal (Adam et al., 2009). Conversely, chronic lymphocytic leukaemia (CLL) is an indolent disease affecting middle-aged to elderly dogs, it is often diagnosed incidentally and characterised by an indolent behaviour (Comazzi et al., 2011). Although canine acute and chronic leukaemias show different clinical behaviour and outcome, leukaemic cells proliferate abnormally, replacing normal bone marrow tissue and contributing to invasion of the extra-cellular matrix (ECM) and resulting blood involvement (Adam et al. 2009).

AIM

To better understand the pathogenesis of canine leukaemia, the present study assessed the expression profiles of the MMP-2, MMP-9, MT1-MMP, TIMP-1, TIMP-2, RECK and VEGF-A genes and the protein levels of MMP-2, MMP-9 and VEGF-A in canine leukaemia.

METHODS

Caseload and classification

Peripheral blood samples were collected in EDTA-containing tubes from dogs with haematopoietic neoplasia and sent for diagnostic purposes by the referring veterinarians to the Department of Veterinary Pathology, Hygiene and Health at the University of Milan and to the Department of Animal Pathology at the University of Turin. Each blood sample was analysed using the Sysmex XT-2000iV (Sysmex Corporation, Kobe, Japan). Peripheral blood and bone marrow smears, when available, were stained with May–Grunwald–Giemsa (Merck KGaA, Frankfurt, Germany) and used to obtain leukocyte differential counts and morphologic evaluations of the blast cells. To determine the immunophenotype, flow cytometric analyses were performed on the peripheral blood samples after erythroid lysis in 2 mL of erythrocyte lysis buffer (9% ammonium chloride, 1% potassium bicarbonate, 0.037% ethylenediaminetetraacetic acid (Sigma Aldrich, Munich, Germany)). The cells were suspended in RPMI 1640 medium (Sigma Aldrich, Munich, Germany) containing 5% foetal bovine serum and 0.2% sodium azide to a final concentration of 1×10^4 cells/µL, and 50 µL of the cell suspensions was used in each tube for the labelling procedures. The details of antibodies are reported in Phase 3 – Section 2. Clinical, clinicopathological and immunophenotypic data were used to classify leukaemia samples as previously described (Gelain et al, 2010). In particular, AL was diagnosed by the presence of: moderate to severe anaemia

and/or thrombocytopaenia, leukocyte morphology suggestive of immature or blast cells, more than 20% of blast cells in the bone marrow, CD34 positivity, and either CD3, CD5, CD4, and CD8 positivity for T-cell ALL, or CD21 and CD79a positivity for B-cell ALL. Samples were classified as acute undifferentiated leukaemia (AUL) when the atypical cells expressed only CD34 and CD45. CLL was diagnosed by the presence of: severe lymphocytosis, monomorphic population of mature lymphocytes, negative serologic titre for *Ehrlichia*, *Leishmania* or any other identifiable cause of lymphocytosis, and either CD3, CD5, CD4 and CD8 positivity for T-cell CLL, or CD21 and CD79a positivity for B-cell CLL. Six peripheral blood samples for immunocytochemical analysis and four peripheral blood samples for quantitative real-time RT-PCR from healthy dogs matched according to age, breed and gender were used as controls. All samples were obtained during an annual general health visit, and informed consent was obtained from all owners.

Sampling procedure

Peripheral blood samples collected in EDTA tubes were firstly submitted to erythroid lysis with 2 mL of erythrocyte lysis buffer. A portion of the sample was used for routine flow cytometric and immunocytochemical analyses, whereas the remainder was placed in polypropylene tubes with 10 parts of RNAlater® solution (Life Technologies, Foster City, CA) for total RNA isolation. At least 0.5 mL of each cell suspension containing of 2×10^6 cell/mL with good viability was required for total RNA isolation. To obtain pure lymphocytes from healthy controls, EDTA blood from the four healthy dogs was pooled. After red blood cells (RBCs) lysis, lymphocytes were separated through granulocytes+monocytes depletion, using MACS separation system. Briefly, after staining with anti-CD11b (clone CA16.3E10, Serotec) and anti-NSA monoclonal (clone CADO48A, VMRD Inc., WA) antibodies (both IgG1 isotype), pelleted leukocytes were magnetically labelled with anti-IgG1 microbeads and passed through MS columns placed in the magnetic field (MACS separator), thus collecting the CD11b-negative NSA-negative fraction. Removing the column from the separator, the magnetical retained (CD11b+NSA+) fraction was eluted in a separate tube. Subsequently, purity (98%) and recovery (99%) of lymphocytes were determined by flow cytometric analysis (BD Accuri C6) as percentage of CD11b-negative cells in the unlabelled fraction and percentage of CD11-positive cells in the labelled fraction, respectively.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA isolation, cDNA synthesis, primers, calibration curve data and the formulation of final value are reported in Phase 3 – Section 2.

Immunocytochemical analysis

The details of the immunohistochemal performance, the antibodies and the labelling valuation are described in Phase 3 – Section 2.

Statistical analysis

The statistical analysis of the gene expression results was performed using the Mann–Whitney test, whereas the immunocytochemistry data were analysed using the Kruskal-Wallis test followed by Dunn's posttest. A non-parametric Spearman correlation analysis was used to determine potential relationships among target genes and between target genes and percentage of neoplastic cells. GraphPad Prism 5 software (San Diego, California, USA) was used for all statistical evaluations. Statistical significance was set at $p<0.05$. Finally, Grubbs' test was used to identify potential outliers.

RESULTS

Clinical results

Peripheral blood samples were obtained from 23 dogs. According to the haematological and flow cytometric diagnosis, 11 dogs were classified as AL: 2 T-cell (CD34+CD5+), 2 B-cell (CD34+CD79a+) and 7 acute undifferentiated leukaemias (CD34+). Eleven dogs were females and one male, with a median age of 8 years (range, 2-12 years). All of the dogs were anaemic, thrombocytopenic associated with leucocytosis. Twelve dogs where classified as T-CLL: 8 CD8+, 1 CD4+, 1 CD21+ and 2 CD4-CD8-). Ten dogs were males and 2 females, with a median age of 10 years (range, 5-13 years). All of the dogs showed leucocytosis and six of them were anaemic. The median percentage values (range) of neoplastic cells in blood were 98.1 (72-100) for AL and 88.2 (72-96) for CLL.

qRT-PCR

The gene expression results are summarised in Table 1 and 2. In lymphocyte control pool the mRNA expression of target genes was generally very low: MMP-2 mRNA was undetectable (n.d.); MMP-9, TIMP-2 and RECK mRNAs were detectable but not quantifiable (n.q.); MT1-MMP, TIMP-1, VEGF-A and VEGF-164 mRNAs were detectable and quantifiable. All selected genes were successfully amplified in ALL and CLL groups, with the exception of MMP-2. All the genes were more expressed in AL and CLL than control lymphocytes (MT1-MMP only for AL), except for TIMP-1 that showed a lower level of gene expression in pathological samples compared with control lymphocytes and MMP-2 that was never amplifiable. Although not statistically significant, the

mRNA levels of MMP-9, TIMP-1, RECK and VEGF-A were higher in CLL compared to AL cases. Instead TIMP-2 and MT1-MMP mRNA levels were significantly higher in AL than CLL. In CLL, significant positive correlations were found between MMP-9 and VEGF mRNAs (*p*<0.01; Spearman r = 0.7545) and between MMP-9 and TIMP-1 mRNAs (*p*<0.05, Spearman r = 0.6182). Moreover, the gene expression results were correlated with the percentage of neoplastic cells in the AL and CLL blood samples. Negative correlations were found between the percentage of neoplastic cells for MMP-9 mRNA (*p*<0.01; 95% confidence interval-CI- "-0.9259 to -0.2760"; Spearman r = -0.7426), for VEGF mRNA (*p*<0.05, 95% CI "-0.8761 to -0.01326"; r = -0.5954), for TIMP-1 mRNA (*p*<0.05, 95% CI "-0.8836 to -0.04644"; r = -0.6165) and for TIMP-2 mRNA (*p*<0.05, 95% CI "-0.8970 to -0.1107"; r = -0.6550). Furthermore, the percentage of neoplastic cells was negatively correlated with MMP-9 mRNA (*p*<0.05; 95% CI "-0.9188 to -0.1530"; r = -0.7002).

Table 1. MMP-9, MMP-2, TIMP-2 and RECK mRNA expression in AL and CLL

Target Genes	**ALL**	**CLL**	***p values***
MMP-9	0.20 ± 0.19	0.59 ± 0.64	*0.1891*
MMP-2	n.d.	n.d.	-
TIMP-2	5.28 ± 3.94	0.51 ± 0.60	*0.0043*
RECK	0.11 ± 0.12	0.23 ± 0.19	*0.1611*

Data are expressed as relative quantification values; arbitrary units (mean ± SD)

n.d. = not detectable

Control lymphocytes = not quantifiable

Table 2. MT1-MMP, TIMP-1, VEGF-A and VEGF-164 mRNA expression in AL and CLL

Target Genes	**ALL**	**CLL**	***p values***
MT1-MMP	1.20 ± 0.91	0.36 ± 0.48	0.0316
TIMP-1	0.39 ± 0.25	0.77 ± 0.72	0.2485
VEGF-A	2.55 ± 3.17	3.19 ± 3.57	0.8777
VEGF-164	4.10 ± 5.05	4.07 ± 4.75	0.7818

Data are expressed as fold changes; arbitrary units (mean ± SD)

Control lymphocytes = 1

Immunocytochemical analysis

Immunocytochemical data for MMP-9, MMP-2 and VEGF-A are summarised in Table 3. The scores for all of the assessed proteins were higher in CLL than in AL (Fig. 1, 2); a significant difference was observed between the control peripheral blood and the leukaemia blood samples (p<0.05).

Table 3. Immunostaining score values for MMP-9, MMP-2 and VEGF-A expressed as the mean (range)

	Control peripheral blood	**AL**	**CLL**	***p values***
MMP-9	0.66 ± 0.51	78.11 ± 64.48	104.1 ± 36.8	*0.0009*
MMP-2	0.16 ± 0.40	56 ± 53.5	71.73 ± 35.51	*0.0008*
VEGF-A	0.5 ± 0.54	21.33 ± 23.62	27.9 ± 15.62	*0.0008*

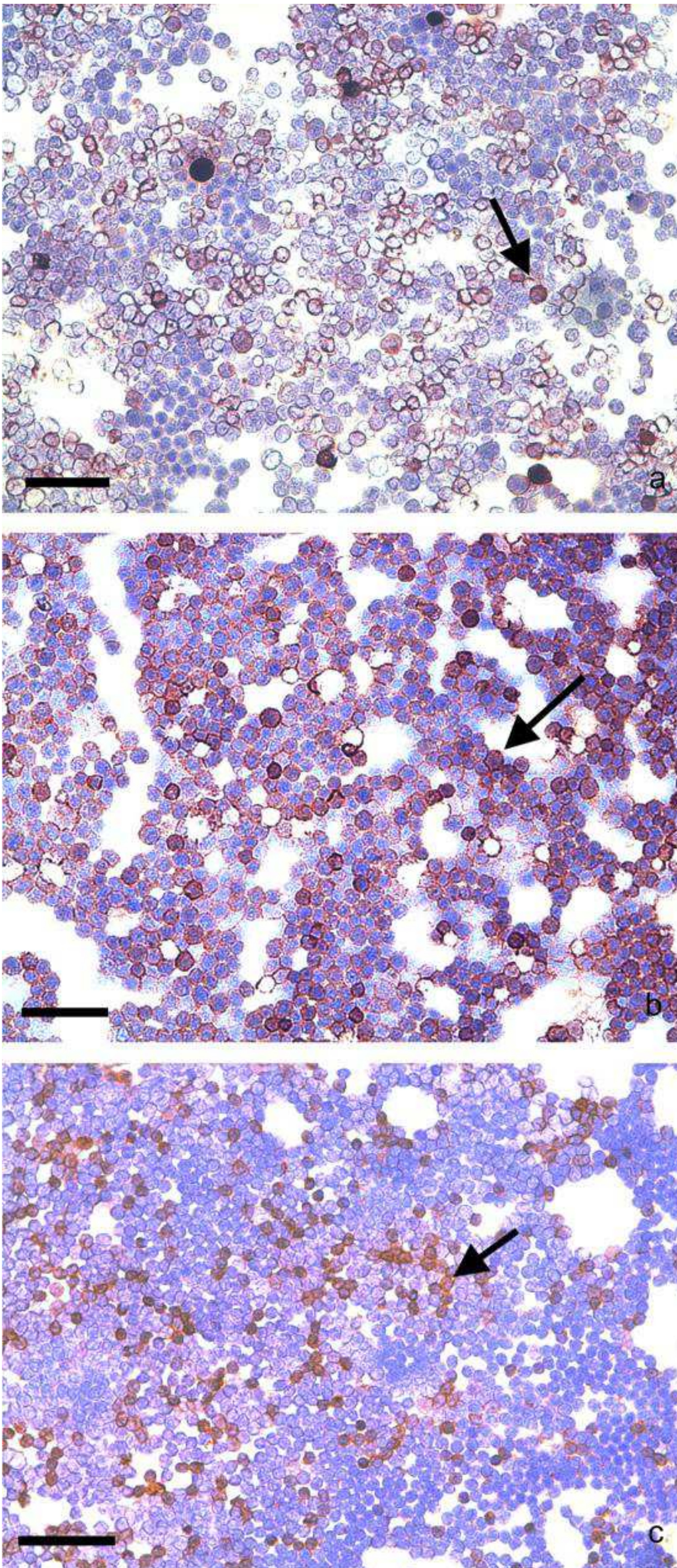

Fig. 1. ALL. Peripheral blood. (a) plasma cells and lymphoid leukaemia cells brown immunostained for MMP-2 (arrow); (b) lymphoid leukaemia cells intensely immunostained for MMP-9 (arrow); and (c) VEGF (arrow). Immunocytochemistry, (bar = 70 lm).

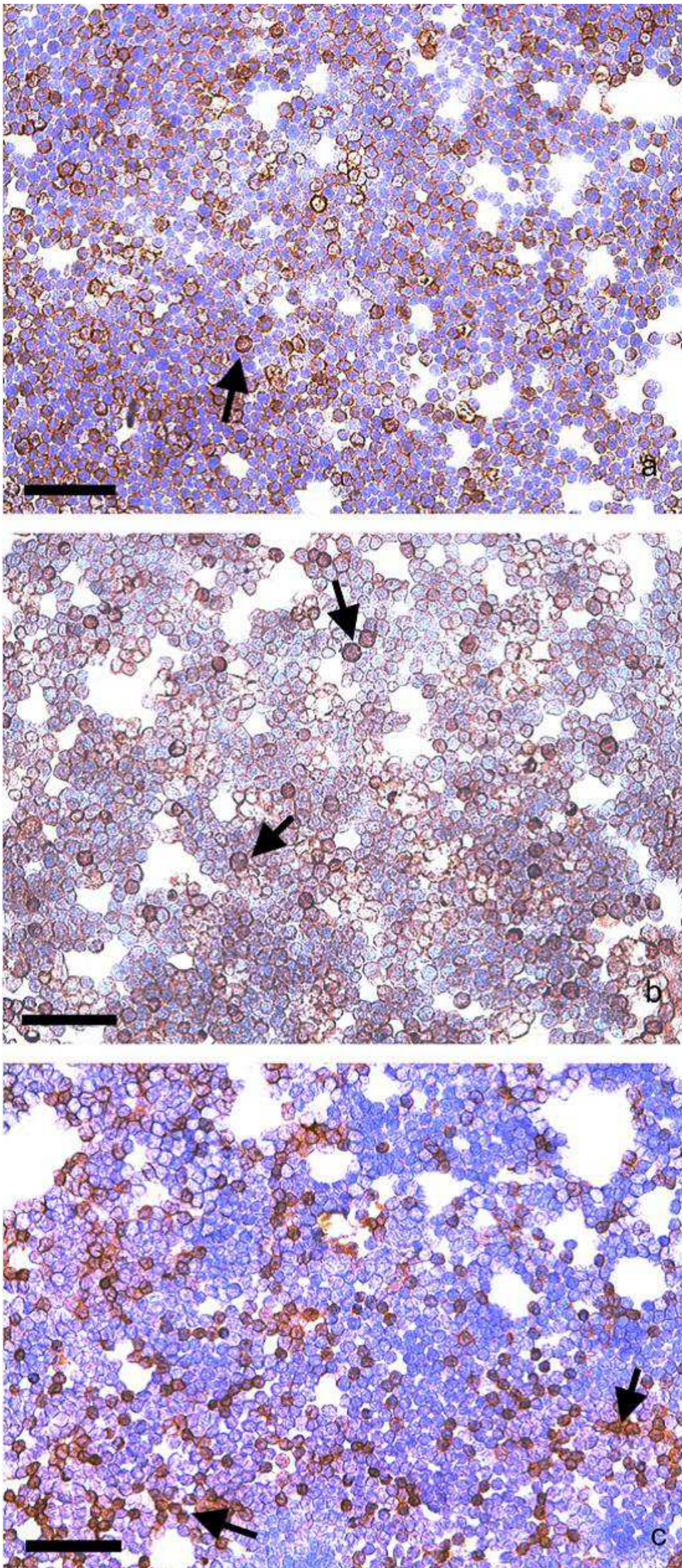

Fig. 2. CLL. Peripheral blood. lymphoid leukaemia cells intensely and diffusely brown immunostained for (a) MMP-2 (arrow), (b) MMP-9 (arrow), and (c) VEGF (arrow). Immunocytochemistry, (bar = 70 lm).

DISCUSSION

Angiogenesis and invasion play an essential role in tumour growth both in solid and hematopoietic tumours in humans (Moehler et al., 2003). Invasion and dissemination of neoplastic cells are regulated by several compounds, in particular MMPs and TIMPs. VEGF has also an influence in this contest and in tumoural neoangiogenesis (Hayashibara et al., 2002, Pedersen et al., 2005, Buggins et al., 2011). In veterinary medicine, different works report the role of these molecules in solid tumours, but there are no data for haematological malignancies. The aim of this study was to investigate different MMPs, their regulators and VEGF in canine acute and chronic leukaemias by assessing their mRNA and protein expression profiles. By definition, AL is the uncontrolled proliferation or expansion of haematopoietic cells that are arrested in an early stage of development. The excessive egress from the bone marrow into the peripheral blood is followed by the infiltration and by the failure of other tissues and organs (Adam et al., 2009). On the contrary, CLL is an indolent disease characterised by the accumulation in the bone marrow of long-lived cells of low proliferative rate, leading to progressive peripheral lymphocytosis (Seelig et al., 2010). In the present study, AL and CLL showed high MMP-9, VEGF-A and VEGF-164 mRNA levels and a positive correlation between MMP-9 and VEGF and between TIMP-1 and MMP-9 was demonstrated in CLL. All the target genes were also screened in pure lymphocytes obtained from healthy control donors. MMP-9 mRNA was not quantifiable and the protein was not expressed in control lymphocytes; whereas higher MMP-9 transcript and protein levels were present in CLL compared to AL. Different authors reported that MMP-9 is necessary for cellular mobility, transendothelial migration and basement membrane invasion by neoplastic cells (Ries et al., 1999, Redondo-Munoz et al., 2008). This may be valid in dog where CLL is characterized by the accumulation in the peripheral blood of neoplastic lymphocytes, which progressively infiltrate the bone marrow and secondary lymphoid tissues. VEGF was highly expressed at the mRNA and protein level in both AL and CLL in respect to control lymphocytes; furthermore, the values for this protein were higher in CLL compared to AL. The hypothesis is that VEGF elevated levels in leukaemic cells could stimulate the growth of new blood vessels in the angiogenetic process and subsequently increase the permeability of endothelium and the cell motility through the vessels leading to the dissemination of cells into organs (Letilovic et al., 2006). Several studies have also indicated that VEGF may promote tumour proliferation (Hayashibara et al., 2002; Poyer et al., 2009; Gehrke et al., 2011). Moreover, a significant positive correlation between VEGF and MMP-9 mRNA levels was demonstrated in CLL samples. It is already known that MMP-9 and VEGF are two

of the most potent factors involved in angiogenesis through different mechanisms. This result provides evidence of the role of MMP-9 in the regulation of neovascularisation in a sort of vicious circle with VEGF. At first, VEGF can stimulate MMP-9 secretion acting as an autocrine factor and subsequently MMP-9 can release different angiogenic factors that bind to ECM, such as VEGF (Bergers et al., 2002; Moehler et al., 2003). In human medicine, both proteins are correlated with the substage of the disease and the risk of progression in CLL; follow-up data were not complete and survival analysis was not performed in this study (Letilovic et al., 2006). TIMP-1 transcript was expressed in both AL and CLL, and a significant positive correlation between MMP-9 and TIMP-1 levels was demonstrated in CLL. MMP-9 is frequently found to be co-expressed with TIMP-1 in canine solid tumours; the same mechanism in CLL was confirmed (Aresu et al., 2011). TIMP-1 is also associated with a more aggressive clinical behaviour in human leukaemias, acting as an anti-apoptotic and differentiation-promoting factor (Kossakowska et al., 1991; Kossakowska et al., 1999; Scrideli et al., 2010). Interestingly, TIMP-2 mRNA expression was no quantifiable in control lymphocytes, whereas the data was 5-fold higher in AL than in CLL. According to literature, TIMP-2 plays several roles in tumourigenesis, and is associated with an unfavourable prognosis and tumour progression (Egeblad et al., 2002). In canine AL samples, TIMP-2 was one of the most highly expressed genes and this might be concordant with the well-known erythroid-potentiating activity and the stimulation of leukaemic cells and fibroblasts growth of this protein (Stetler-Stevenson et al., 1992; Hayakawa et al., 1994; Corcoran et Stetler-Stevenson, 1995). Unfortunately, no data for TIMP-2 protein were available in this work but the negative correlation with MMP-2 observed demonstrate that the biological role of natural inhibitor of MMP-2 proteolytic activity is still active. MT1-MMP transcript was significantly different between AL and CLL. MT1-MMP is usually localised on the cell surface, where it regulates different members of the MMPs family, such as pro-MMP-2 and pro-MMP-13, creating a wider proteolytic repertoire on the cell surface. In particular, the MMP-2 activity is regulated by the formation of a ternary complex that consists of the C-terminal domain of pro-MMP-2, TIMP-2 and MT1-MMP. The comparable results for TIMP-2 and MT1-MMP suggest the presence of this mechanism in AL. However, MT1-MMP alone can also degrade various ECM macromolecules, including collagen, fibronectin and laminin (Egeblad et al., 2002). Hotary et al. (2003) reported that the expression of MT1-MMP is crucial for cancer cell growth in a 3D collagen-based matrix, suggesting that MT1-MMP has an important role in cancer invasion. CLL, AL and control lymphocytes showed an undetectable level of MMP-2 mRNA. MMP-2 expression is rarely observed in human ALL (Kuittinen et al., 2001;

Scrideli et al., 2010). Paradoxically, MMP-2 protein expression was identified in leukaemic cells by immunocytochemical analysis but not in lymphocytes in control blood. This discrepancy can be explained by the fact that MMP-2 protein activity is mainly regulated at the post-transcriptional level (Brown et al., 1990). In different solid tumours it has been demonstrated the catalytic activity of MMP-2 with insignificant expression of MMP-2 mRNA (Caenazzo et al., 1998; Aresu et al., 2011). Interestingly, an inverse correlation between the percentage of neoplastic cells and the expression of all the genes was identified in CLL. Similar result was obtained for MMP-9 in AL. The residual population (i.e. neutrophils and monocytes) might influence the expression levels of MMP-9, MT1-MMP, VEGF and TIMPs, proportionally. It's already known that neutrophils and monocytes are able to express physiologically different MMPs, it remains still not fully understood the participation of these cells in the tumour microenvironment (Letilovic et al., 2006). In conclusion, significant relationships were demonstrated between MMP-9 and VEGF and between MMP-9 and TIMP-1 in CLL. MMP-9, TIMP-1 and VEGF were highly expressed in CLL and TIMP-2 and MT1-MMP in AL. In this study a first evidence of the potential role of these molecules was provided in pathogenesis of canine leukaemia. Further studies are needed to clarify the detailed molecular mechanisms involved in the signal transduction of MMP-2, MT1-MMP and their specific regulators/inhibitors. Moreover, MMP-9 and VEGF could be further investigated in plasma during haematological malignancies in dogs and correlated to the clinical data. Understanding their roles may help in designing new therapeutic strategies for canine leukaemias.

5.4. SECTION 4: Expression of Matrix Metalloproteinases, Tissue Inhibitors of Metalloproteinases and Vascular Endothelial Growth Factor in Canine Lymphohematopoietic malignancies cell lines

BACKGROUND

Several studies in human lymphohematopoietic malignancies were performed in vitro to elucidate the possible involvement of growth factors and proteinases without the confounding influence of stromal and endothelial cells found in lymphoma tissue specimens. MMPs have been investigated in human cell lines of B and T-cell origin (Kossakowska et al., 1999; Stetler-Stevenson et al., 1997). More recently VEGF and its receptors were detected in human cell lines of cutaneous T-cell lymphoproliferative disorders (Pedersen et al., 2012) and in precursor B-cell acute lymphoblastic leukaemia cell line (El-Obeid et al., 2004). To date, little is known about these molecules in canine tumour cell lines: in vitro MMPs were detected in canine melanoma (Docampo et al., 2011), macrophage/monocytic tumour (Puff et al., 2009) and osteosarcoma (Loukopoulos et al., 2004), whereas VEGF and its receptors in canine mastocytoma (Rebuzzi et al., 2007).

AIM

The aim of this study is to consider the expression of various MMPs and their inhibitors, VEGF and VEGFR-2 at both the mRNA and protein levels in four different canine lymphohematopoietic malignancies cell lines. These analyses were performed to elucidate the possible involvement of these molecules without the confounding influence of microenvironment in the pathogenesis of canine lymphoid neoplasms.

METHODS

Cell lines

Canine cell lines used in this study include: B-cell lymphoma cell line CLBL-1 (Rütgen et a., 2010),T-cell lymphoma cell line OSW (Kisseberth et al., 2007), T-lymphoblastoid cell line CL-1 (Momoi et al., 1997) and B-cell leukaemia cell line GL-1 (Nakaichi et al., 1996) (Fig. 1). The cell culture conditions were described previously by Rütgen et al. (2010).

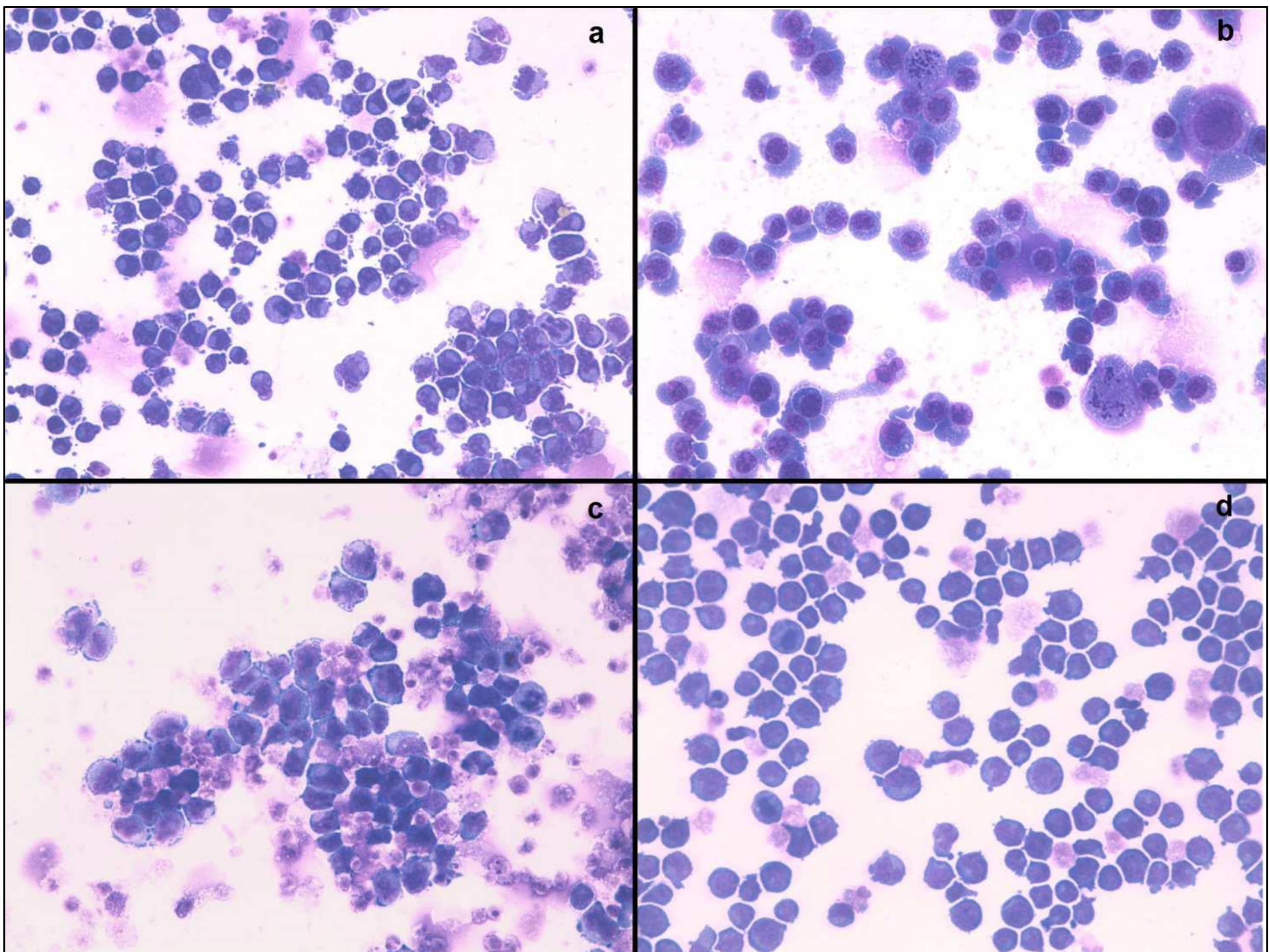

Fig. 1. Canine lymphohematopoietic malignancies cell lines. A) B-cell lymphoma cell line CLBL-1. B) T-cell lymphoma cell line OSW. C) T-lymphoblastoid cell line CL-1. D) B-cell leukaemia cell line GL-1. (Haematoxylin-Eosin - 400X)

Sampling procedure

The cells were harvested at the exponential growth phase, washed 3× in RPMI 1640 medium (Sigma Aldrich, Munich, Germany) with serum (as an internal control – conditioned medium presents slight amounts of MMPs) or serum-free, aliquoted at a concentration of 2 × 10^6cells/mL and incubated for 24 hours at 37°C and 5% CO_2. The cell-conditioned media (supernatants) were collected to perform gelatin zymography, whereas two samples were obtained from each cell pellet for immunocytochemical analysis and total RNA extraction. For both protein and mRNA expression, at least 0.5 mL of each cell suspension containing of 2 × 10^6 cell/mL of good viability was required.

Quantitative real-time RT-PCR (qRT-PCR)

The total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To avoid genomic DNA contamination, on-column DNase digestion with the RNase-Free DNase set was performed. cDNA syntheses were done using 250 ng RNA and the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Primers, calibration curve data and the formulation of final value are reported in Phase 3 – Section 2.

Immunocytochemical analysis

The protein expression levels of MMP-2, MMP-9, MT1-MMP, TIMP-2, VEGF-A and VEGFR-2 were evaluated by immunocytochemistry. The details of the immunohistochemal performance are described in Phase 3 – Section 2. The pertinent antibody details are summarized in Table 1. The intensity and the index of immunoassayed tumour cells were assessed for each antibody. Antibodies labelling index was defined as the percentage of tumour cells displaying immunoreactivity in ten randomly selected fields at 400x magnification. An intensity score of 0 was given when no staining was detected, a score of 1 denoted moderate staining and a score of 2 represented strong staining. The total score for each examined field was obtained by multiplying the intensity score by the index. A final ratio was obtained after averaging the ten selected fields. The image analysis system included an Olympus BX51 microscope and a software analysis (analySIS, Soft imaging system, Münster, Germany).

Table 1: Details of antibodies

Antigen	Source	Clone	Dilution	Manufacturer
MMP-9	Human	C-TERM	1:200	Millipore S.p.A, Milan, Italy
MMP-2	Human	Ab-7	1:100	Thermo Fisher Scientific Inc., Kalamazoo, Michigan, USA
TIMP-2	Human	MAB 3310	1:500	Chemicon (Millipore)
MT1-MMP	Human	AB8221	1:200	Chemicon (Millipore)
VEGF-A	Human	A-20 – sc:152	1:100	Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA
VEGFR-2	Human	Flk-1 (A-3): sc-6251	1:50	Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA

MMPs analysis using gelatine zymography

MMP-2 and MMP-9 activity was studied by zymography. A 1:4 dilution was made from supernatants of each cell line into sample buffer, and 60 µL of the diluted sample was subjected to electrophoresis on an 8% SDS-PAGE gel co-polymerized with 0.1% gelatine. Other details about gelatine zymography performance and the quantification of bands are reported in Phase 1.

RESULTS

Gene expression

The gene expression results for each cell line are summarised in Table 2. A higher MMP-9 mRNA expression was observed in OSW compared to the other cell lines; whereas MMP-2 was undetected in all cell lines. MT1-MMP and TIMP-1 expressions were observed in T-cell line (OSW), but no results were obtained in CLBL-1, CL-1 and GL-1. VEGF-A and VEGFR-2 transcripts were detected in all cell lines, but expression levels were higher in GL-1. Whereas B-cell leukaemia cell line (GL-1) presented higher TIMP-2 mRNA expression respect to CLBL-1, OSW and CL-1, which exhibited expression values close to 0. RECK was detected in the four cell lines, but T-cell line (OSW) presented a higher expression profiling.

Table 2. MMP-9, MMP-2, MT1-MMP, TIMP-1, TIMP-2, VEGF-A, VEGF-164 and RECK mRNA expression in the four cell lines.

	CLBL-1	**OSW**	**CL-1**	**GL-1**
MMP-9	0,76	1128,35	0,05	0,03
MMP-2	0,00	0,00	0,00	0,00
MT1-MMP	0,04	4,11	0,01	0,00
VEGF-A	4,87	1,42	3,11	9,42
VEGF-164	7,99	3,18	4,68	20,53
TIMP-1	0,72	2,38	0,00	0,72
TIMP-2	0,00	0,82	0,00	28,54
RECK	4,32	19,56	6,84	5,96

Data are expressed as relative quantification values (mean ± SD)

Immunocytochemistry

Immunocytochemical results are summarised in Table 3. The immunostaining scores for the target proteins were variable. CLBL-1 and OSW were positive immunostained for all markers (Fig. 2).

Table 3. MMP-9, MMP-2, MT1-MMP, VEGF-A, VEGFR-2 and TIMP-2 protein expression in the four cell lines.

	CLBL-1	**OSW**	**CL-1**	**GL-1**
MMP-9	88	190	0	100
MMP-2	44	0	0	90
MT1-MMP	62	100	0	92
VEGF-A	80	30	0	0
VEGFR-2	68	90	92	0
TIMP-2	46	198	100	198

Data are expressed as immunostaining scores

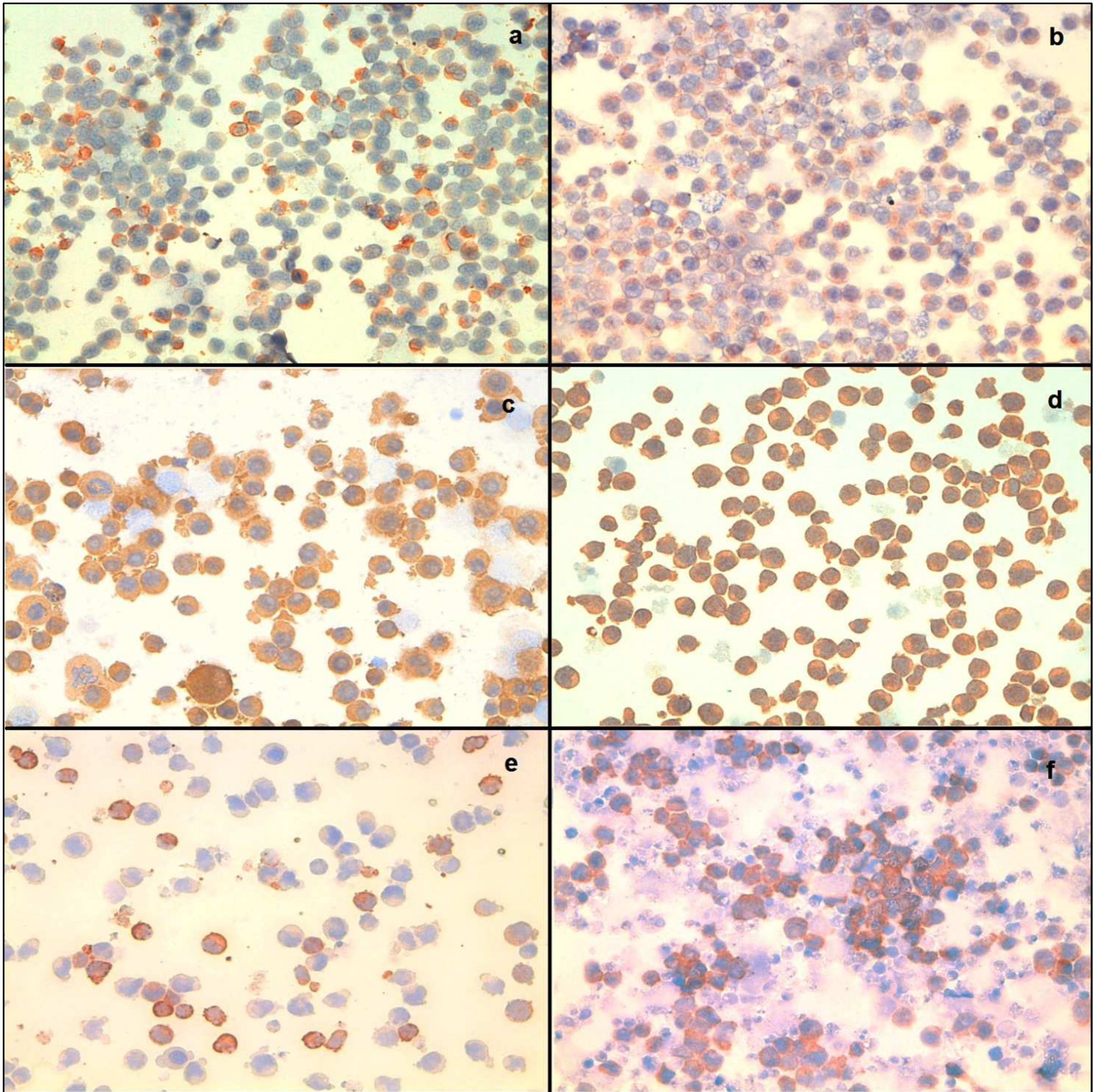

Fig. 2. Immunostaining in four canine lymphohematopoietic malignancies cell lines. A) Intense MMP-2 immunopositivity in CLBL-1. B) Moderate MT1-MMP immunopositivity in OSW. C) Intense MMP-9 immunopositivity in OSW. D) Intense TIMP-2 immunopositivity in GL-1. E) Intense VEGF immunopositivity in CLBL-1. D) Intense VEGFR-2 immunopositivity in CL-1. (Immunohistochemistry, 400×).

Gelatin zymography

Catalytic activities of both latent and mature form of MMP-2 and MMP-9 were undetected in all cell lines except for pro-MMP-9, which presented an identifiable band (Fig. 3).

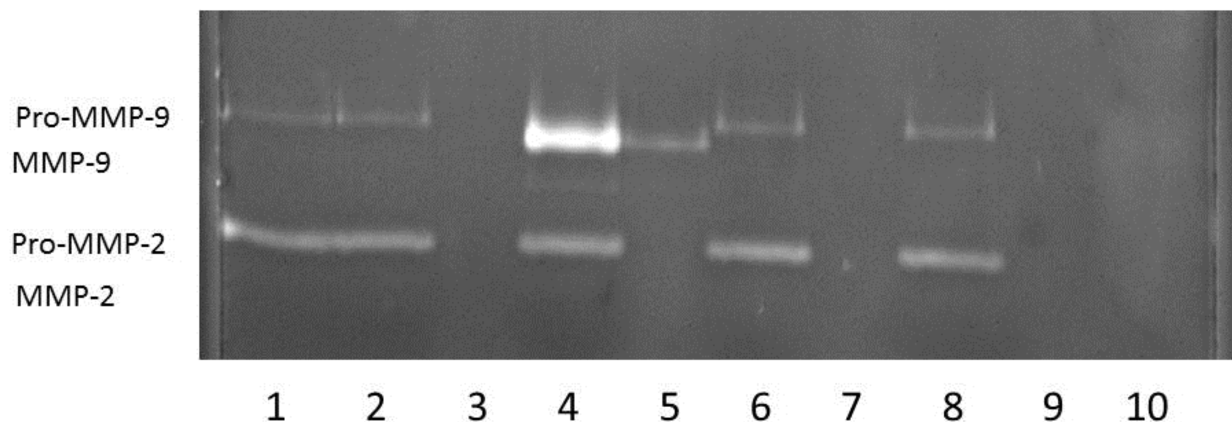

Fig. 3. Representative MMP zymography from canine Lymphohematopoietic malignancies cell lines, grown in the last 24 hours in medium with serum (as internal control) and serum-free. Lanes 1: positive control. Lane 2 and 3: CLBL-1 with serum and serum-free. Lanes 4 and 5: OSW with serum and serum-free. Lane 6 and 7: CL-1 with serum and serum-free. Lane 8 and 9: GL-1 with serum and serum-free. Lane 10: negative control.

DISCUSSION

Because the bone marrow and lymphatic organs are predominant sites of tumour accumulation in hematologic malignancies, it was initially believed that angiogenesis, in association with mechanisms of tumour invasion and growth, would not be as relevant in these disorders (‘‘liquid tumours’’) as it is in solid tumours. However many studies showed a relation between various hematologic malignancies and changes in the angiogenic profile (Letilovic et al. 2006). In this study different molecules *in vitro* were assessed, which are involved in the tumour invasion and angiogenesis to exclude the influence of microenvironment. The involvement of the MMPs and TIMPs in development of the metastatic phenotype in hematopoietic neoplasms both *in vitro* and *in vivo* is well established (Kossakowska et al., 1999; Stetler-Stevenson et al., 1997). The expression of MMP-9 using the sensitive zymogram technique didn’t show significative results for the four cell

lines in the present study. A considerable amount of the zymogram detection of pro-MMP-9 was only detected in the T-cells lymphoma (OSW), in association with its high mRNA and immunolabelling expression level. Biologically the role of this enzyme in normal lymphocyte is to permit the migration from the vascular compartment to sites of inflammation, contributing significantly to events associated with T-cell transmigration across the subendothelial basement membrane (Stetler-Stevenson et al., 1997). This process could be comparable to invasion of structures by lymphoma or leukemic cells, indicating that MMP-9 may play an important role correlated to the immunophenotype. Furthermore previous studies demonstrated that lymphoid cells *in vitro*, also using Matrigel (reconstituted basement membrane), may be induced to produce MMP-9 (Kossakowska et al., 1998). MMP-2 was not detected by RT-PCR and gelatin zymography, whereas the immunostaining expression was observed in B-cell lymphoma cell line (CLBL-1) and B-cell leukaemia cell line (GL-1). This discrepancy between different methods, also observed *in vivo*, might be explained by the fact that MMP-2 protein activity is mainly regulated at the post-transcriptional level (Brown et al., 1990). In different solid tumours it has been demonstrated the catalytic activity of MMP-2 with insignificant expression of MMP-2 mRNA (Caenazzo et al., 1998, Aresu et al., 2011). The same discrepancy was identified in CLBL-1 and GL-1 in regard to MMP-9 results: lymphoid cells may produce MMPs upon interaction with basement membrane components, such as laminin, type IV collagen, or heparan sulfate proteoglycan, or in response to other tissue-specific interactions (eg, interaction with endothelial cells) that are not present under *in vitro* tissue culture conditions. Moreover the identification of the gelatinases mRNAs is, however, not synonymous with the presence of their enzymatic activity, as mRNA codes for the protein, which at first is present in the inactive latent form. The enzymatic activity of gelatinases is dependent on the activation of their latent forms (Kossakowska et al., 1998). High levels of TIMP-2 RNA transcript and protein expression in B-cell leukaemia cell line (GL-1) were demonstrated, whereas TIMP-1 presented a similar trend with MMP-9, supporting the hypothesis that MMP-9 and TIMP-1 may act in concert in canine T-cell lymphoma. Although the gelatinases may be expressed under different culture conditions, one would expect regulation of TIMP-1 and TIMP-2 mirroring the one of MMP-9 and -2, due to the inhibitory action of the TIMPs. Therefore, the constitutive expression of the TIMPs without gelatinases suggests that they may have a growth factor activity (Kossakowska et al., 1991), similar to the demonstrated erytroid-potentiating activity (Stetler-Stevenson et al., 1997).

Interestingly MT1-MMP RNA transcript and protein reflected MMP-9 trend, with high values in OSW. Like MMP-9, MT1-MMP is known to have an important functional role in tumour invasion; it degrades ECM macromolecules, cytokines, and chemokines and stimulates the mobility and migration of neoplastic cells. MT1-MMP is also known to promote tumour angiogenesis, degrading the fibrin matrix that surrounds newly formed vessels (Egeblad et al., 2002). In regard to VEGF, the principal molecule and the isoform -164 were always expressed and presented similar trends with a higher mRNA expression level in GL-1 respect to the other cell lines. On the contrary in GL-1, VEGF-A protein expression was not observed. This could be explained by the fact that tumour growth in chronic lymphocytic leukaemia (CLL), as well as in other hematologic malignancies, is strongly influenced by angiogenesis, primarily through powerful cytokine interactions, both paracrine and autocrine. These interactions are found between at least three subsets of cells found in bone marrow: endothelial cells, nearby stromal cells and malignant CLL cells, which produce angiogenic factors such as VEGF, but only the first two could trigger the activation of these factors (Letilovic et al., 2006). In this study T-cell lines (OSW and CL-1) expressed higher levels of RECK mRNA than their B-cell counterparts (CLBL-1 and GL-1), as it was observed *in vivo*. It is hypothesised that RECK expression inversely correlates with that of MMPs due to its tumour-suppressing activity (Noda et al., 2003). These results indicate that this simple direct relationship cannot hold true in the context of canine lymphomas. In humans, RECK down-regulation has been correlated with tumour progression and angiogenesis (Noda et al., 2003), but, in literature, no data have demonstrated the biologic behaviour of RECK in haematopoietic malignancies. In dogs, the mRNA expression levels of RECK in some tumour tissue samples were significantly lower than those in normal tissue samples (Takagi et al., 2005; Aresu et al., 2011). To my knowledge, this is the first study investigating MMPs and VEGF in canine hematopoietic cell lines by using transcript and protein analyses. The potential role of MMP-9, TIMP-1 and -2 in tumour invasion was confirmed. Moreover it was demonstrated that cancer cells in primary tumours have a strict interaction with their supportive microenvironment, which triggers the secretion of growth factors and proteases. This process allows amplifying activation the signals in the cascade that results in the establishment of an activated stroma that promotes malignant tumour growth. Further efforts should be directed towards new therapeutic approaches in the treatment of canine lymphoid malignancies focused on tumour microenvironment.

5.5. SECTION 5: Expression of Platelet-derived Growth Factor and its receptors in canine Lymphoma

BACKGROUND

Platelet-derived growth factor (PDGF) and its receptors play a significant role in human hematopoietic malignancies. In Hodgkin's lymphoma, it has been shown that the majority of Reed-Sternberg cells express PDGFR-α and are dependent by PDGFR signalling, whereas normal B-cells or B-cells in NHL do not express PDGFR and do not rely on its signalling (Renne et al., 2005). Karabatsou et al. (2006) showed that primary central nervous system lymphomas express PDGFR-α in the majority of investigated cases, whereas PDGF-A is expressed less frequently. A study on NHL showed PDGF-B level decrease after radiotherapy, with a possible predictive significance for response to treatment and recurrence (Ria et al., 2008), whereas Duşe et al. (2012) assessed PDGF-Rs protein expression in NHL. Interestingly, Ho et al. (2005) analysed the expression of PDGF in B-cell lineage, showing that abundant PDGF-A, rather than PDGF-B, was expressed in normal B cells. No difference was reported for the expression of PDGF-A and PDGF-B between patients with reactive lymphoid hyperplasia and B-cell chronic lymphocytic leukaemia (B-CLL) and B-cell lymphoma. Among the patients with B-CLL, the expression of PDGF-B was stronger than the expression of PDGF-A. They concluded that PDGF-A it is important in B cell differentiation and proliferation, considering its expression in all stages of B lymphocyte differentiation, whereas the expression of PDGF-B and PDGFR-β suggests that autocrine signalling of PDGF may be important in malignant transformation of B-CLL. At present time no data are available on the expression of PDGF and its receptors in canine hematopoietic malignancies.

AIM

To understand the possible correlation between the expression of PDGF-A and -B, in association with the expression of PDGFR-α and -β in canine lymphoma, their gene expression profiling will be defined in dogs with B-cell and T-cell lymphoma and with reactive hyperplasia, also focusing on possible prognostic and predictive role of these molecules. To combine and compare work in vitro and in vivo, PDGF-A, -B, PDGFR-α and -β mRNA expression was also assessed in four different canine lymphohaematopoietic tumour cell lines.

METHODS

Cell lines

Canine cell lines used in this study include: B-cell lymphoma cell line CLBL-1 (Rütgen et a., 2010),T-cell lymphoma cell line OSW (Kisseberth et al., 2007), T-lymphoblastoid cell line CL-1 (Momoi et al., 1997) and B-cell leukaemia cell line GL-1 (Nakaichi et al., 1996) (Fig. 1). The cell culture conditions, sampling procedure and generated cDNA are described in Phase 3 – Section 4.

Case Selection and Tissue Sampling

Forty-two dogs underwent complete staging work-up, including physical examination, complete blood cell count, peripheral blood and bone marrow aspirate, thoracic radiography and abdominal ultrasound. Flow cytometric and cytological analysis of nodal or cutaneous fine-needle aspirate were described previously by Aresu et al. (2012). Furthermore histological examination and immunohistochemistry of nodal or cutaneous biopsy were assessed. Fresh tissue samples were also obtained and excised tumour lesions were immediately divided into aliquots and stored under diverse conditions for different analytical techniques. The dogs underwent surgery due to evident disease, and the explicit consent of the owner was obtained. Five control lymph nodes were obtained from pathogen-free adult dogs, with consent of owners. For histological examination and immunohistochemistry, the tissue was formalin-fixed and paraffin embedded. For RNA isolation, aliquots of approximately 100 mg were immersed in RNAlater® solution (Applied Biosystems, Foster City, CA) and stored at -20°C until use. Owners of dogs with lymphoma were offered to treat their animals with multidrug chemotherapy, consisting of doxorubicin, vincristine, cyclophosphamide, L-asparaginase and prednisone (Simon et al., 2006). In these dogs, fresh tissues were collected at three time points (at diagnosis, at the end of treatment and at relapse).

Histological and immunohistochemical examination

Samples were fixed in 4% buffered formalin, embedded in paraffin and cut at 3-μm sections. For histological examination, slides were stained with haematoxylin and eosin. An antibody panel for diagnosis of lymphoid neoplasms was evaluated by immunohistochemistry. The primary antibody incubation step was performed by an automated system for all antibodies (Ventana Medical Systems). A monoclonal Mouse Anti-Human CD3 (Clone F7.2.38, Dako, Atlanta, GA, USA, T cells; diluted 1 in 100), a monoclonal Mouse Anti-Human CD5 (Clone CD5/54/F6, Dako, T cells; diluted 1 in 100), a monoclonal Mouse Anti-Human CD79αcy (Clone HM57, Dako, all stages of B-cells;

diluted 1 in 100), a CD20 Epitope Specific Rabbit Antibody (RB-9013-P, Thermo Fisher Scientific Inc, Cheshire WA7 1TA, UK, mature B cells; diluted 1 in 800) were used. The Ventana ES automated immunohistochemistry system was used for the remainder of the staining procedure, including the incubation with a biotinylated anti-mouse secondary antibody, the diaminobenzidine substrate and a haematoxylin counterstain. Negative control slides were incubated with isotype-matched immunoglobulin in parallel with each staining batch to confirm the specificity of the antibodies. The image analysis system included an Olympus BX51 microscope. Samples were classified based on WHO Classification of tumours of domestic animals (Valli et al., 2002).

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA isolation and the formulation of final value are reported in Phase 1. First-strand cDNA was synthesised from 300 ng of total RNA using Superscript II (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The generated cDNA was used as the template for quantitative real-time RT-PCR (qRT-PCR) in a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) using standard PCR conditions. The qRT-PCR reactions consisted of 5 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Life Technologies, Carlsbad, CA), 0,3 µl of forward and reverse primers (10 µM) (the primer combination and final concentrations were optimized during assay setup) and 2,5 µl of diluted (1 to X) cDNA. The primers, shown in Table 1, were designed using Primer Express 2.0 (Applied Biosystem, Life Technologies, Carlsbad, CA). Calibration curves using a 7-fold serial dilution of a cDNA pool revealed PCR efficiencies near two and error values < 0.2. Canine transmembrane BAX inhibitor motif containing 4 (CGI-119) was chosen as reference gene for the absence of pathological state dependent differences in mRNA expression, as reported in Aricò et al. (2012).

Table 1: Primer sequences used for qRT-PCR amplification

Genes	Accession Numer	Primer Sequence (5'-3')
PDGF-A	NM_001190172.1	F: TTTGGAAGCAAGTCTGAGAGCC R: TGGCCTCCTCAATGCTTCTT
PDGF-B	NM_001003383.1	F: CCGAGTTGGACCTGAATTTG R: GTCTTGCACTCAGCGATCAT
PDGFR-α	AY525124.2	F: TTTCCCTTGGCGGCACAC R: GTCAGGCTTGGCCATCCG
PDGFR-β	NM_001003382.1	F: CACGCCTCTGACGAGATTTATG R: CTCGAGAAGCAGCACCAGCT

Statistical analysis

The statistical analysis of the gene expression results was performed using the Mann–Whitney test. A non-parametric Spearman correlation analysis was used to determine potential relationships among target genes. A non-parametric paired t test data (Wilcoxon signed rank test) was used to identify differences in gene expression between the samples collected in the three time points. GraphPad Prism 5 software (San Diego, California, USA) was used for all statistical evaluations. Statistical significance was set at $p<0.05$. Finally, Grubbs' test was used to identify potential outliers.

RESULTS

Clinical results

Thirty-three dogs with lymphoma and 9 with reactive hyperplasia were enrolled. In Table 2 lymphoma subtypes are reported, based on cytological and flow cytometric, and histological and immunostochemical evaluation. At the end of treatment 4 dogs were in relapse (2 B-cell lymphomas and 2 T-cell lymphomas), whereas 9 dogs were in remission status (8 B-cell lymphomas and one T-cell lymphoma). Two out of 8 B-cell lymphoma in remission status experienced a relapse during the course of the study.

Table 2: Classification of lymphoid tumours

Immunophenotype	Subtype	Number
B-cell	Diffuse Large B-cell Lymphoma (DLBCL)	19
	Marginal zone Lymphoma (MZL)	2
	Follicular Lymphoma (FL)	1
T-cell	Peripheral T-cell Lymphoma (PTCL)	5
	Cutaneous Lymphoma (CL)	3
	Lymphoblastic Lymphoma (LL)	3

qRT-PCR

The gene expression results for healthy control lymph nodes, reactive hyperplasia, B-cell and T-cell lymphomas and cell lines are summarised in Table 3. PDGF-A, PDGFR-α and PDGFR-β mRNA levels in the healthy control lymph nodes were significantly higher than in lymphomas. A significantly higher PDGF-B mRNA expression was observed in T-cell lymphomas compared to B-cell lymphomas. PDGF-B mRNA expression was significantly higher in cutaneous lymphomas respect to control lymph nodes. Interestingly PDGF-A, PDGFR-α and PDGFR-β mRNA levels in the healthy control lymph nodes, reactive hyperplasia and lymphoma in remission status (2,12 ± 0,91; 2,9 ± 1,7; 1,81 ± 0,84 respectively) were similar; PDGF-B transcript amount was similar in reactive hyperplasias and lymphomas in remission status (1,83 ± 0,93), but lower in control lymph nodes. mRNA levels of all genes in cell lines were not quantifiable or very low, with the exception of PDGF-A and PDGFR-α mRNA expression in OSW and PDGF-A mRNA levels in GL-1. Comparing results at diagnosis and at the end of treatment in B-cell lymphomas, significant correlations were found between PDGFR-β and both PDGF isoforms at diagnosis, whereas in second time point between PDGF-A and PDGFR- α (Table 4). In Table 5 mRNA expression of all genes are reported in Diffuse Large b-cell Lymphoma (DLBCL) cases at relapse and after the end of treatment. Similar values at diagnosis and relapse were found whereas expression levels were higher but not significant at the end of chemotherapy. With a paired t test (Fig. 1) it was evident a significant expression trend ($p < 0.05$) of the 8 DLBCL cases of both PDGF isoforms and their receptors at the diagnosis and at the end of therapy, being higher mRNA levels at the second time point.

Table 3: PDGF-A, PDGF-B, PDGFR-α and PDGFR-β mRNA expression in reactive hyperplasia, B-cell and T-cell lymphoma

	PDGF-A	PDGF-B	PDGFR-α	PDGFR-β
B-cell lymphoma	0,84 ± 0,45	0,55 ± 0,36	0,78 ± 0,49	0,47 ± 0,34
DLBCL	0,83 ± 0,41	0,56 ± 0,36	0,75 ± 0,46	0,44 ± 0,26
nodal subtype	0,91 ± 0,76	0,52 ± 0,43	0,97 ± 0,77	0,67 ± 0,73
T-cell lymphoma	0,79 ± 0,48	2,26 ± 1,96[c]	0,67 ± 0,36	0,68 ± 0,35
PTCL	0,69 ± 0,34	2,13 ± 2,25	0,66 ± 0,48	0,52 ± 0,38
CL	1,2 ± 0,6	3,48 ± 1,61[d]	0,9 ± 0,22	1,08 ± 0,11
LL	0,56 ± 0,48	1,26 ± 1,63	0,47 ± 0,13	0,56 ± 0,14
reative hyperplasia	1,95 ± 1,13	2,01 ± 1,36	2,29 ± 1,54	1,55 ± 0,85
control	1,93 ± 0,73[ab]	0,91 ± 0,51	3,52 ± 0,71[ab]	1,35 ± 0,50[ab]
CLBL-1	n.q.	n.q.	5,01E-03	n.q.
OSW	2,22	3,54E-03	3,46	1,05E-03
CL-1	0,55	n.q.	n.q.	n.q.
GL-1	1,54	n.q.	9,48E-03	3,26E-02

Data are expressed as the mean ± standard error

n.q. = not quantifiable

[a, b, c, d] Significant differences between control lymph nodes and B-cell lymphomas, control lymph nodes and T-cell lymphoma,s B-cell lymphomas and T-cell lymphomas, cutaneous lymphomas and control lymph nodes, respectively (Mann–Whitney test, $P < 0.05$).

Table 4: Correlation between different genes in B-cell lymphoma

	PDGFR-α	PDGFR-β
PDGF-A	at end of treatment r=0,76 p<0,036	at diagnosis r=0,58 p<0,0041
PDGF-B	/	at diagnosis r=0,63 p<0,0014

Table 5: PDGF-A, PDGF-B, PDGFR-α and PDGFR-β mRNA expression in two DLBCL cases in relapse after treatment

	PDGF-A		
	diagnosis	end of treatment	relapse
case 1	0,96	1,37	0,41
case 2	0,96	1,37	0,73
	PDGF-B		
	diagnosis	end of treatment	relapse
case 1	0,55	3,50	0,51
case 2	0,30	1,99	0,35
	PDGFR-α		
	diagnosis	end of treatment	relapse
case 1	0,44	2,23	0,49
case 2	0,45	5,20	0,74
	PDGFR-β		
	diagnosis	end of treatment	relapse
case 1	0,20	1,57	0,23
case 2	0,12	2,80	0,28

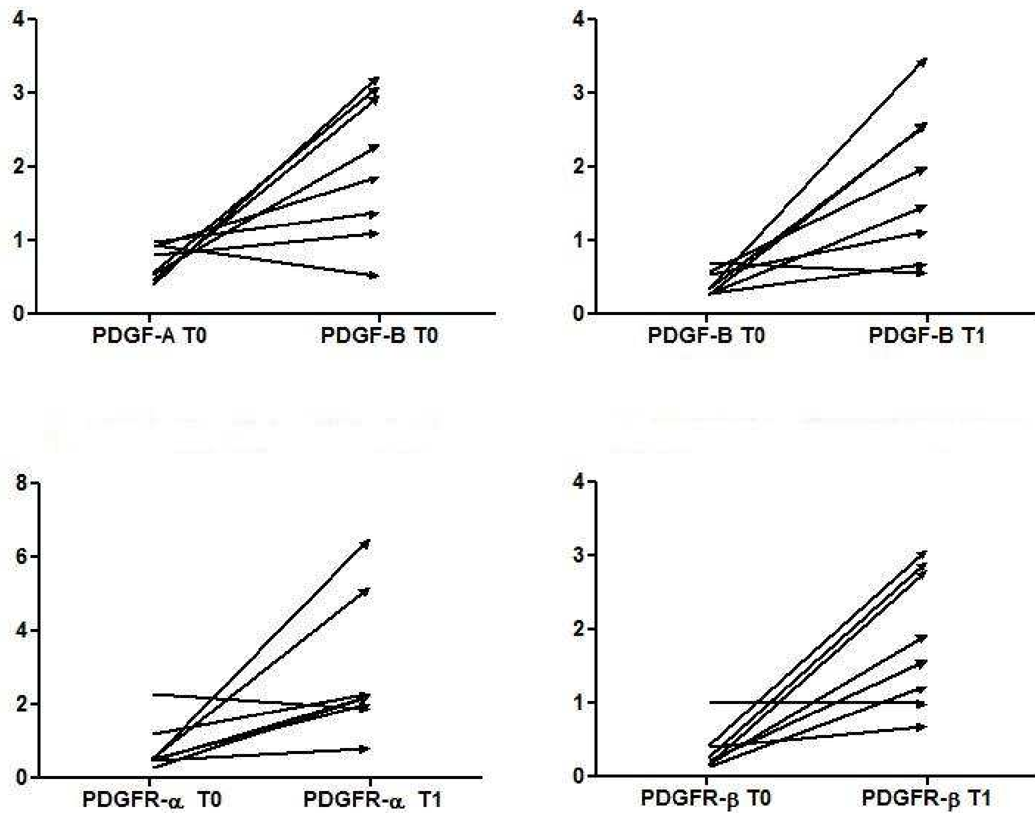

Fig. 1. Paired t test in two time point. Graphs showing the expression trend of PDGF and its receptors at the diagnosis (T0) and at the end of treatment (T1) in 8 cases of DLBCL.

DISCUSSION

Several works have clarified the possible involvement of PDGF in human hematopoietic malignancies (Ho et al., 2005; Renne et al., 2005; Karabatsou et al., 2006; Ria et al., 2008), supporting the existence of an autocrine loop and the notion that it contributes to the uncontrollable growth of some malignant cells. Moreover, overexpression of PDGF and its receptors seems to be relevant in the pathogenesis of NHL (Karabatsou et al. 2006; Ria et al., 2008; Duşe et al., 2012). Considering no data available at present about PDGF and PDGFRs expression in canine lymphoma, in the present study gene expression profiling of these molecules was assessed. The results obtained show that PDGF-A, PDGFR-α and PDGFR-β mRNA levels in the healthy control lymph nodes were significantly higher than in lymphoma-affected dogs. In human it is well documented that PDGFRs are poorly expressed in hematopoietic cells (Toffalini and Demoulin, 2010). Moreover PDGF-A, rather than PDGF-B, is expressed in normal B cell and there are no difference in the expression of PDGF-A and PDGF-B between patients with reactive lymphoid hyperplasia and patients with B-cell lymphoma (Ho et al., 2005). Interestingly, in the present study, in T-cell lymphoma PDGF-B was significantly higher respect to B-cell counterpart: in particular cutaneous lymphoma subtype showed the most considerable mRNA expression levels. From the literature, cell lines corresponding to the advanced stage of a cutaneous T-cell lymphoma showed enhanced expression of PDGF; in addition, post-thymic T-cell malignancies, derived from activated T-cells, may produce and secrete growth factor, like PDGF (Su and Kadin, 1989). Moreover an autocrine PDGF-BB/PDGF b-receptor loop was found to mediate survival of large granular lymphocyte leukaemia of both T- and NK-cell origin (Yang et al., 2010). The present data showed that canine T-cell lymphomas, in particular cutaneous subtype, may represent a new and important source of PDGF-B and that PDGF-B signalling could also be important in the pathogenesis of this neoplasia, contributing to its aggressive growth characteristics and different clinicopathologic behaviours. Considering the findings obtained in cell lines, PDGF-A and PDGFR-α expression in OSW (T-cell lymphoma cell line) and PDGF-A expression in GL-1 (B-cell leukaemia cell line) were the only highlighted. Therefore T-cell line showed the expression of a different isoform of PDGF (PDGF-A) and the interaction with its specific receptor. To explain this opposite trend in T-cell lymphoma line respect to vivo model, it is important to note that different isoforms of PDGF give different cellular effects and responses, which is explained by their different interactions with α- and β-receptors (Heldin et al., 1998). Studies on PDGF in human hematopoietic cell lines are few and showed expression of PDGF-B and PDGFR-β in B-cell chronic lymphocytic leukaemia,

suggesting that autocrine signalling of PDGF may be important in the malignant transformation of this neoplasia (Ho et al., 2005), whereas PDGF-A was evident in pre-B acute lymphocytic leukaemia cell lines (Tsai et al., 1994). Comparing gene expression results at diagnosis and at the end of treatment in B-cell lymphoma, significant correlations were found between PDGFR-β and both PDGF isoforms A and B at diagnosis, whereas at the end of chemotherapy between PDGF-A and PDGFR-α. The two PDGF receptor types mediate similar, but not identical, cellular responses. After activation, both receptors stimulate cell proliferation and rearrangement of actin filaments. The β-receptor mediates a potent chemotactic response. Moreover, PDGF protects cells against conventional and programmed death (Heldin et al., 1998). Therefore it is possible that this different combination between PDGF isoforms and receptors leads to several cellular effects and responses which are unique during the disease. Further investigations are needed to deeply assess the ligand-induced activation of receptors and PDGF signalling in dogs. Interestingly a significant expression trend ($p < 0.05$) of all molecules investigated at the diagnosis and at the end of therapy was showed in DLBCL which experienced a remission status, with higher expression at the end of treatment. This finding confirms in B-cell lymphomas the low expression of PDGF and its receptors, whereas in remission status, in which lymph node tissue goes to a hyperplastic or atrophic condition, PDGF mRNA transcript amount increases. This biological behaviour can explain the similar gene expression profiling of all molecules in the healthy control lymph nodes, reactive hyperplasia and B-cell lymphoma in remission status, then the two DLBCL dogs in relapse at the end of treatment. In conclusion, PDGF-B is probably involved in the pathogenesis of canine T-cell lymphoma, in particular of cutaneous subtype, and the co-expression of PDGFs and PDGFRs in different time point during disease suggests a functional autocrine and/or paracrine loop of growth stimulation, which gives different cellular effects and responses. Further investigations are needed to deeply assess the role of PDGF and its receptors in canine lymphoma, especially considering protein analysis, which will be the further step. This evaluation is thought to identify the cellular type expressing these molecules (tumour cells and/or stromal cells), the intensity and the percentage of immunoassayed cells, to compare with gene expression findings. PDGF-B may represent new therapeutic target in canine T-cell lymphoma, but future studies should collect more clinical outcome data to further understand a potential prognostic role of this molecule.

6. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

There is abundant evidence that crosstalk between tumour cells and their reactive stromal cells contributes to, or is even required for, tumour formation and progression. Tumour cells create a supportive microenvironment by secreting growth factors, proteases and their inhibitors, whose imbalance leads to the degradation of ECM, resulting in the release of growth factors, bound to the matrix, and of ECM molecular fragments. The secretion can act both in autocrine and paracrine manners to the stroma, inducing angiogenesis, invasion and proliferation of tumour cells. In addition to recruitment, proliferation and activation of stromal inflammatory cells and fibroblasts are activated, secreting further growth factors and proteases. This cycle permits to amplify these signals in the cascade resulting in the establishment of an activated stroma that promotes malignant tumour growth. Advantages to targeting the stroma include the fact that these cells are not genetically unstable as cancer cells, and are therefore less likely to develop drug resistance. Several exciting success stories have already been presented in the clinical targeting of tumour stroma (Mueller and Fusenig, 2004). It is important to note that “Normalization” of the stromal environment should be able to slow or even reverse tumour progression. “Normalize” microenvironment consists in a phenotypic reversion of a malignant and invasive to a non-invasive pre-malignant tumour phenotype. Blocking angiogenesis is considers one possible approach, in which the activated stromal compartment of malignant neoplasm is normalized, blood vessels acquire an intact basement membrane, and fibroblast activation is downregulated. As a consequence of the downregulation of MMPs or other proteases and reduced ECM turnover in the stroma, an intact basement-membrane zone is re-established and stromal collagen is reformed. As a result of this normalized stromal compartment, the malignant and invasive growth of tumour reverts to a pre-malignant dysplastic phenotype (Skobe et al., 1997). However, there are also some disappointments in targeting the stroma for cancer therapy in human, as reported in the results on clinical testing of MMP inhibitors: no efficacy in patients suffering from advanced stages of cancer; severe intolerable side effects and worsening the prognosis for the patient. To overcome these problems, in human oncology, the development of more specific inhibitors is now underway (Mueller and Fusenig, 2004).

This research project has brought out several important results, which upgrade the background on the tumour progression and the role of microenvironment in veterinary oncology. These findings are:

- the involvement of MMP-2, MT1-MMP, MMP-9 in canine mammary tumours, with an emphasis on the stromal compartment;
- the role of MMP-9 and VEGF-A in the progression and malignancy of canine cutaneous mast cell tumours;
- MMP-9, MT1-MMP, TIMP-1 and VEGF influence in T-cell lymphomas and in dogs with a more advanced disease, highlighting the correlation with phenotype and grading;
- the potential role of MT1-MMP and TIMP-2 in the pathogenesis of canine acute lymphoblastic leukaemia;
- the influence of residual normal leukocytes in the expression of MMP-9, MT1-MMP, VEGF and TIMPs in chronic lymphocytic leukaemia ;
- the observed discrepancy between in vivo and in vitro model in lymphoma and leukaemia to highlight the importance of microenvironment;
- the possible interaction of PDGF-B in canine T-cell lymphoma, in particular in cutaneous subtypes, and the suggested functional autocrine and/or paracrine loop of growth stimulation confirmed by the co-expression of PDGFs and PDGFRs at different time point during disease.

In the last years, the tumour vasculature has also become an attractive target for therapy in veterinary oncology. Therefore, the future in canine targeted therapy in mammary tumour, mast cell tumour, lymphoid leukaemia and lymphoma, will consider combination of drugs that target different aspects of the activated stroma with cytotoxic therapies that are directed against neoplastic cells.

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