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IRON SERUM STATUS, SERUM VEGF AND OXIDATIVE STRESS PATTERN
EVALUATION IN CANINE CANCER PATIENTS, FOCUSING ON MAST CELL TUMOURS

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ABSTRACT

The aim of our this study was to investigate the role iron serum status, serum vascular endothelial growth factor and oxidative stress pattern in canine cancer patients with particular attention on cutaneous mast cell tumour. Mast cell tumors (MCTs) are the most common cutaneous tumor in the dog, accounting for 16% to 21% of cutaneous tumors (Bostock,1986; Finnie,1979; Rothwell, 1987; Brodey,1970). Wide variation is seen in the histologic pattern of canine MCT, and the histologic grade has been clearly established as a strong prognostic factor that is highly predictive of biologic behavior and clinical outcome. It has been demonstrated that mast cells and mast cells tumour can express, produce and release the Vascular endothelial growth factor (VEGF) has been implicated to contribute tissue edema through its effect on vascular permeability (Paturno, 2009; Mederle, 2010). Studies performed in vitro and in vivo (rats) on Mast cells, showed also how VEGF expression was regulated by hypoxiainducible factor-1a (HIF-1a) activation through the phosphatidylinositol 3-kinase (PI3K)–HIF-1a pathway (Lee, 2008). Systemic hypoxia produces an inflammatory response characterized by increases in reactive O2 species (ROS), venular leukocyte-endothelial adherence and emigration, and vascular permeability. The results showed that mast cells could play a key role in hypoxia-induced inflammation and suggested that alterations in the ROS-nitric oxide balance may be involved in mast cell activation during hypoxia (Steiner, 2003). Studies performed in vitro on mast cells have also demonstrated the role of the antioxidant barrier, especially of the vitamin E, in prevent degranulation of mast cells and proliferation of tumour cell lines (Gueck, 2002; Reiter, 2003). Most of these study have been performed in vitro and, except for the VEGF (which has been often investigated in the last years for his role in the anticancer target therapy in veterinary medicine), there are just few data on the role of oxidative stress pattern and iron profile in canine patients affected by mast cell tumour, as

well as in several other neoplastic disease. These data are just assumed from human studies where both in vitro and in vivo a network between iron serum profile, VEGF and oxidative stress pattern has been established as they are involved in the progression of neoplastic diseases as well as in the body's response to the tumour. So the aim of this study was to investigate the role of this network in canine oncology patients through different tumour time and clinical substage focusing on mast cell tumour.

INTRODUCTION

Neo-angiogenesis plays a crucial role in tumour development, spreading and growths of metastatic disease. Although several different molecules and pathways are responsible of this complex event, the Vascular Endothelial Growth Factor (VEGF) is recognized as one of the main pro-angiogenetic factors (Folkman, 1992). In the early stage of tumour development, we observe a first a-vascular stage in which the tumour obtains oxygen and nutrients through the process of diffusion and simple diffusion (Grupta, 2003), and this stage the tumour is quiescent (Dvorak, 1986). A tumour is composed by a heterogenic population of cells and some of the them, due to molecular changes, increased characteristic of malignancy, inhibition of onco-suppressor genes (such as RAS, Raf and p53) (Chiarugi V., 1998), or due to any inflammatory or traumatic event acquiring an angiogenetic phenotype. The acquisition and expression of this phenotype allows the formation of an adequate vascular bed and the consequent exponential growth of the tumour. The angiogenesis is supported and promoted by the VEGF secreted by the tumor cells and also by stromal cells, platelets, the immune system (mast cells, macrophages, T lymphocytes) and vascular cells, particularly in hypoxic areas. Hypoxia leads to the production of HIF, hypoxia-inducible factor, a transcription factor that stimulates the transcription of the VEGF encoding gene. Angiogenesis, as well as be an essential process for the growth of primary tumor, has a special importance in enabling metastatic spread. In fact the new vessels, because of their thin wall with poor development of the basement membrane, are easily traversed by the neoplastic cells, representing a convenient access route to the bloodstream (Papetti, 2002).

Iron is essential for life, is a cofactor of several oxide reductive enzymes, and is involved in oxygen transport and storage and in DNA synthesis. A small amount of the body iron is exchanged with the outside world and much of it is recycled; iron resulting by physiological cells' death is collected by macrophages, re-entering the life cycle. Body iron levels are then set only during absorption through the small intestine and the small amount of iron that is lost must be replenished daily to maintain balance (Finch, 1982). If not, either because of an excessive loss or because the amount absorbed is not enough, we observe an iron deficiency that, over the time, will lead to the development of anemia. On the other hand, if the amount of iron entering the body exceeded the its requirements, this will be slowly accumulated in the body and particularly in the liver, leading to the development of iron overload. Due to the oxide-reducing capacity of the iron, this event will may lead to the formation of oxygen reactive species (ROS) that can damage cellular (Gomme, 2005).

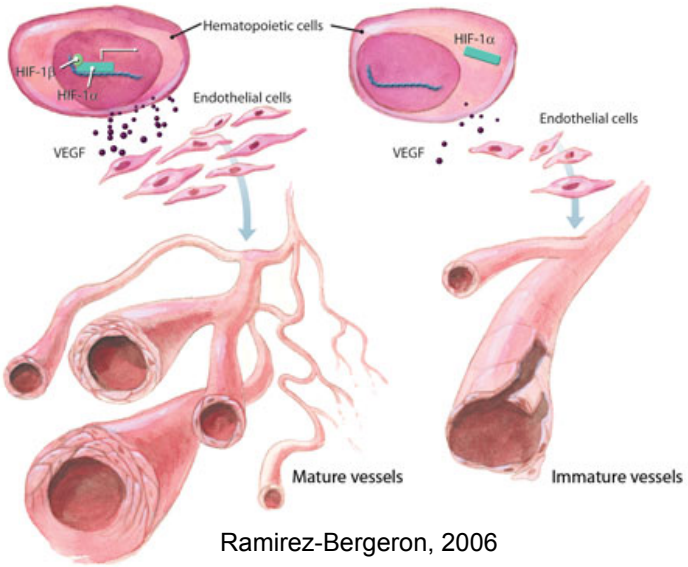
Vitamin E is a powerful antioxidant capable of blocking the lipid peroxidation of cell membranes, breaking the covalent bonds formed between ROS and fatty acids of the cell membrane. Iron deficiency increase HIF-1a and VEGF serum concentration and it is a promoter of angiogenesis, suggesting that systemic iron deficiency could play an important role in tumor progression (Eckard, 2010).

1. Vascular Endothelial Growth Factor (VEGF)

1.1. VASCULOGENESIS AND ANGIOGENESIS

The formation of new blood vessels can develop from two different process, vasculogenesis and angiogenesis. Although the vasculogenesis is the process of de-novo formation of vessels from haemangioblast stem cells which occurs in the early stages of embryogenesis (Risau, 1995; Patan, 2004), endothelial cell precursors are also found within the bone marrow of adults and are recruited due to trauma or ischemia (Zammaretti, 2005). During angiogenesis, the new vessels are sprouting from endothelial cells of preexisting vessels and despite angiogenesis is one of the main cause of solid tumor growth and progression it is also involved in inflammatory process (Asahara, 1997; Djonov, 2000; Peichev, 2000; Djonov, 2003; Patan, 2004) showing also important physiological function, such as wound healing (Ruiter,.1993; Paavonen, 2000).

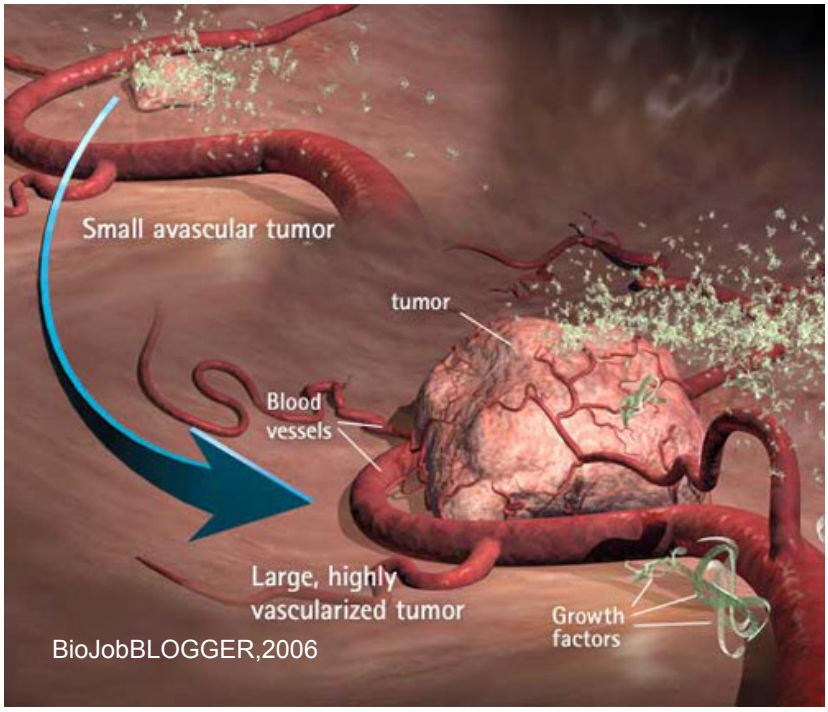
In adults the vascular system is almost quiescent, except during physiological process like wound healing, hair growth and the menstruation (Salven, 2001, Ferrara, 2001). In these circumstances the angiogenic mechanism is closely controlled and remains active for relatively short periods; The imbalance in demand and supply of oxygen and nutrients, as for example chronic inflammatory conditions (rheumatoid arthritis, inflammatory bowel disease), diabetes (diabetic retinopathy), inflammatory and



Ramirez-Bergeron, 2006

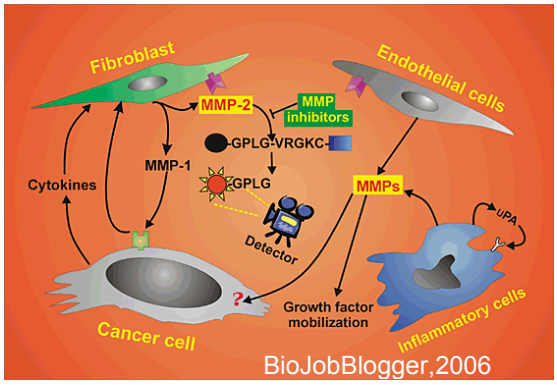
hyperproliferative skin diseases such as psoriasis, endometriosis and tumours (Salven, 2001) results in sprouting of new capillaries from pre-existing vessels through angiogenesis.

The study of the ability of a neoplastic tissue to induce formation of a new vascular network was one of the main objectives scientific researches over the past decade. Understanding this mechanism has allowed in human medicine to support the chemotherapy "standard" therapies, which aim to inhibition of tumor growth and treatment of both the primary tumor, both of possible metastatic spread. The tumor angiogenesis is a process formation of a vascular network within a tissue neoplastic and departing from it. Solid tumors are strictly dependent on the formation of new vessels, "a fabric cancer without a vascular bed can not grow larger than the diameter of 1-2 mm (Folkman, 1992). In pre-neoplastic vascular tissue seldom exceeds 2 mm in size (eg, carcinoma in situ) and, at this stage the cells can get oxygen and nutrients needed for survival and growth, and eliminate catabolites produced through a process of simple passive diffusion (Gupta, 2003). Besides this extension, this mechanism is not sufficient to trophic ensure the growth of tumors, also, the state of hypoxia that is established, leads via activation of p53, apoptosis (Cotran, 1999). At this stage, a tumour may remain quiescent for a long time, reaching a balance between the number replicating cells and those who are dying, until the time in which some of them switch to an angiogenic phenotype (List, 2001).



The angiogenic switch is a phenomenon caused by a rearrangement of gene expression, responsible of the balance between the factors stimulating and inhibiting angiogenesis. The acquisition of this phenotype by some tumour cells allows the formation of a proper vascular growth and the secondary tumour growth facilitating the metastatic process.

The local vessel growth is induced by the release of angiogenic soluble factors by the involved tissue activating endothelial cells. The interactions between angiogenic factors and their receptors provide signals for cell migration, proliferation and differentiation forming new capillaries. As previously mentioned, angiogenesis is a complex process, characterized by a cascade of events such as an initial vasodilatation accompanied by an increase of vascular permeability and degradation of surrounding matrix, allowing activated endothelial cells in



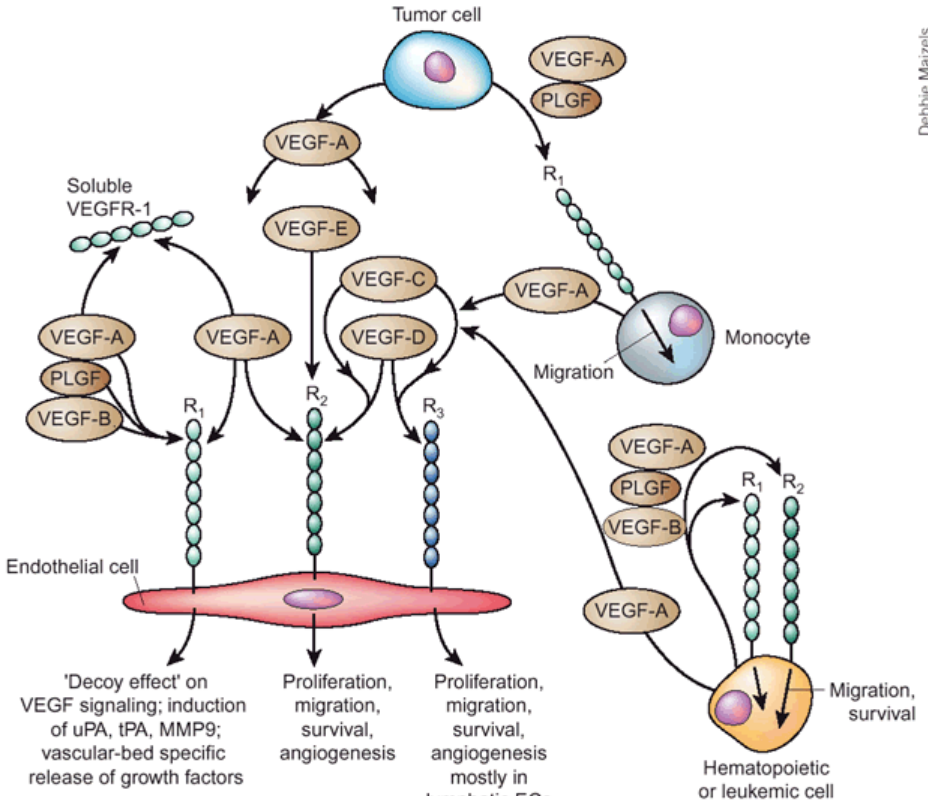
proliferation and migration forming new vessels. The vessels derived from the sprouting of endothelial cells are supported by a network of differentiated endothelial cells and peri-matrix. Subsequently, at a stage of maturation and remodelling, these new vessels take place in the formation of a vascular network. The invasion of endothelial cells and their migration require the presence of various enzymatic activities such as plasminogen activator, metalloproteinase and various cysteine proteases **(**Mignatti, 1996**).** The expression of protease genes is induced by cytokines and angiogenic factors such as of the basic fibroblast growth factor (FGFb) and vascular endothelial growth factor (VEGF), while proteolysis is stimulated by the activation of pro-proteases on the one hand, and in decreasing levels of endogenous inhibitors proteolytic enzymes. The activated endothelial cells express integrin-like $\alpha_v\beta_3$ e $\alpha_v\beta_5$ that allow the migration through the degraded matrix, followed by their proliferation. Subsequently, the endothelial cells of new born capillaries synthesize a new basement membrane. In addition, the stabilization of new capillaries is accompanied by recruitment of pericytes and smooth muscle cells (Crocker, 1970). This process is regulated by the platelet derived growth factor (PDGF) (Lindahl, 1997; Hellström, 2001). The process of angiogenesis is tightly controlled by a dynamic balance between pro-angiogenic factors and anti-angiogenic factors (i.e. Thrombospondin-1). In physiological conditions the balance between pro angiogenic and anti angiogenic factors shifts in favour of the latter.

There is ample evidence for recognizing VEGF as the main pro-angiogenic factor (Ferrara, 1999). The VEGF stimulates endothelial cells to degrade their basement membrane, migrate and express integrins $\alpha_v\beta_3$ e $\alpha_v\beta_5$. Furthermore, VEGF is able to stimulate in vitro survival and proliferation of endothelial cells and the tube formation by endothelial cells themselves.

1.2. VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF is a dimeric glycoprotein of about 40 kDa and is a potent mitogen stimulating the proliferation and migration of endothelial cells, expression of metalloproteinases (Pufe, 2004) and the formation of endothelial fenestrations (Monaghan-Benson, 2009). VEGF also induces the increase of vascular permeability causing extravasal accumulation of fibrin (the substrate for the activity of endothelial cells and tumor cells) and finally, interacts with the cells of the immune system (including natural killer) inducing the expression of adhesion molecules.

In mammals, the VEGF family comprises seven members: VEGF-A (usually the one considered as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and PlGF (placental growth factor). Alternative splicing results in different variants of VEGF that include $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ $VEGF_{206}$, and several other forms. The solubility of these variants depends on the affinity for heparin. The $VEGF_{206}$ and $VEGF_{189}$ are soluble forms that do not bind tightly to heparin and, therefore, remain sequestered in the extracellular matrix. The $VEGF_{165}$ binds heparin with lower affinity, but may also be associated with the matrix, while the $VEGF_{121}$ lacks the ability to bind heparin and is therefore the more soluble form of VEGF. The VEGF-A is involved in the increased vascular permeability and angiogenesis. The VEGF-B is currently being studied for its role in tumor progression and seems not to be linked to angiogenesis, whereas VEGF-C and VEGF-D are widely studied for their role in angiogenesis and linfoangiogenesis in cancer (Ferrara, 2003).

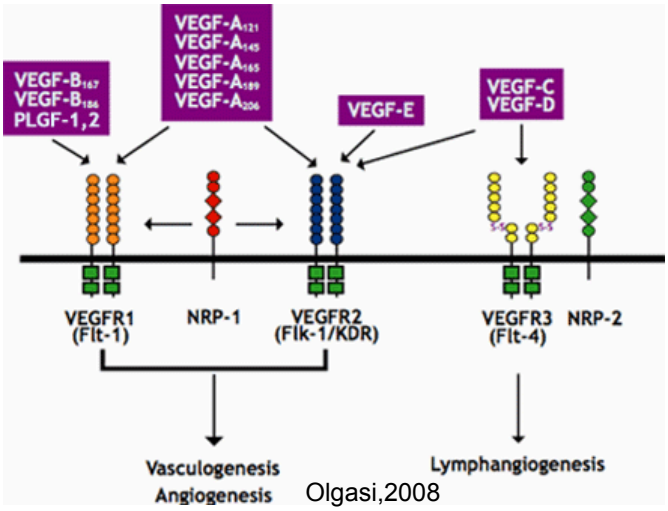


Ferrara , 2003

1.3. VEGF RECEPTORS

Angiogenesis is controlled by paracrine signals and most of them are produced by the binding between small molecules (i.e. VEGF) and transmembrane receptor tyrosine kinase (RTK). The human VEGF protein binds with high affinity with two classes of receptor tyrosine kinase: a) flt-1 (fms-like tyrosine-kinase) is expressed on vascular endothelial cells, confirming the primary action of VEGF on vascular endothelium (Fong, 1995). b) KDR (kinase domain region) known also as flk-1 (fetal liver kinase-1) is identified on endothelial cells, monocytes and tumor cells (Shalaby, 1995) showing affinity for $VEGF_{165}$ but not $VEGF_{121}$. As members of the RTK family, both the receptors are organized into three domains: an extracellular domain consisting of

seven extracellular region similar to immunoglobulins (Ig-like), a transmembrane and an intracellular domain responsible of the intracellular tyrosine kinase activity. Flt-4 is also a member of the RTK family and binds VEGF-C. The interaction with both receptors (flt-1 and KDR) is essential to induce the full spectrum of VEGF biological activity. The cascade of events involved in the transduction signal induced by VEGF (which begins with the autophosphorylation and dimerization of the receptors upon interaction with the ligand), is followed by a succession of phosphorylations that leads to the activation of alternative pathways (i.e. Ras) (Grugel, 1995; Fong, 1995; Shalaby, 1995; Merenmies, 1997). VEGF shows different biological effects depending on the binding receptor; although flt-1 and KDR are highly homologous they activate distinct pathways sending different signals to the endothelial cells. Numerous experiments using cell lines transfected with flt-1 and KDR genes concluded that their expression is essential for most of the VEGF biological effects: while the interaction between VEGF and flk-1/KDR is followed by proliferation of endothelial cells, no mitogenic effects were observed after VEGF bound with flt-1. Other studies showed that the interaction of VEGF with flt-1 activates phosphatidylinositol pathway and, for this reason, it may be involved in monocytes migration. The expression of high affinity VEGF receptors during the early stages of embryonic development (hemangioblasts), suggests a role in vasculogenesis and angiogenesis and is one of the first events of the differentiation of endothelial cells. It has been also reported how conditions of chronic oxygen deficits, in which angiogenesis is a more appropriate response, VEGF receptors are



Olgasi,2008

increased and thus potentially facilitate VEGF action. Indeed, hypoxia increases VEGF binding sites in cultured bovine retinal endothelial cells and increased mRNA levels of both KDR/Flk and Fit in the lung of rats exposed to hypoxia. (Takagi, 1996.)

1.4. REGULATION OF GENE EXPRESSION OF VEGF

Several mechanisms are involved in the regulation VEGF gene regulation. An important role is played by tissue O_2 tension, as evidenced by reversible mRNA levels of VEGF under hypoxic conditions in vivo and in vitro. The increased expression of VEGF mRNA is mainly mediated by the transcription factor HIF-1 (hipoxia-inducible factor 1), which binds to a recognition site in the promoter region of the VEGF gene. (Harris,2002) The hypoxic induction of VEGF expression is mediated through adenosine a2 receptors and consequent increase of intracellular cAMP through the activation of the translation related to c-src. Increased levels of VEGF mRNA in response to hypoxia is due to an increased stability of the mRNA by attachment of a protein induced by hypoxia at the 3 'non-coding mRNA. Is reported that the VEGF mRNA is regulated at the transcriptional and post-transcriptional level also by glucose deficit. Moreover, several cytokines are able to operate indirectly an up-regulation of VEGF mRNA, resulting in protein synthesis and secretion of vascular endothelial growth factor. Transcription Growth Factor-a (TGF-a), TGF-b, epidermal growth factor (EGF), the mediators of the inflammatory response such as interleukin (IL)-1b and prostaglandin E2 are able to induce the expression of VEGF, suggesting the latter's participation in inflammatory processes. It has been also reported that Insulin Growth Factor 1 (IGF-1), in addition to its proliferative effect, can induce the expression of VEGF by tumor cells. Activation of protein kinase C, protein kinase A, Ras oncogene

and overexpression of v-raf or v-src is also related to VEGF over expression (Rak, 1995). Moreover the cell cycle regulatory proteins such as p53 and suppressor gene Von Hippel-Lindau (VHL) have been also described as VEGF up-regulatory factors. (Mukhopadhyay, 1997). The increased level of concentration of VEGF also leads to over-expression of the receptors Flt-1and KDR on endothelial cells. Later, after the vascular development the expression of VEGF is reduced gradually (Penn, 2008).

In the 1970s Judah Folkman stated that tumors were dipendent on blood vessels for their growth eh expansion (Folkman, 1971). This concept of angiogenesis is the formation of new blood vessels from pre-existing vessels. The 1980s saw the advent in several laboratories of a tumor-derived protein inducing angiogenesis, called vascular endothelial growth factor A (VEGFA) (Ferrara, 1989; Leung, 1989; Plouet, 1989), and one vascular permeability factor (VPF), which increased permeability (Keck, 1989; Senger, 1986). It was later revealed that these two proteins were one and the same, and was upregulated in all solid tumors acting via its receptors found mainly on endothelial cells in blood vessels (Rennel, 2009)

1.5. VEGF, OXYGEN REACTIVE SPECIES (ROS) AND MICROVASCULAR PERMEABILITY

VEGF greatly enhances microvascular permeability; however, the molecular mechanisms controlling VEGF-induced permeability remain unknown. Endothelial permeability is mediated by two pathways: the transcellular pathway and the paracellular pathway. In the transcellular pathway material passes through the cells, whereas in the paracellular pathway fluid and macromolecules pass between the

cells. The paracellular pathway is regulated by the properties of endothelial cell-cell junctions (Dejana, 2004; Muller, 2003; Vestweber, 2008).

Treatment of microvascular endothelial cells with VEGF led to an increase in reactive oxygen species (ROS) production. Additionally, treatment with VEGF caused ROS-dependent tyrosine phosphorylation of both vascular-endothelial (VE)-cadherin and β-catenin (Monaghan-Benson, 2009). VE-cadherin is an endothelial cell-specific adhesion molecule that connects adjacent endothelial cells (Gotsch, 1997; Corada, 1999). Disruption of VE-cadherin is sufficient to disrupt intercellular junctions (Corada, 1999; Carmeliet, 1999; May, 2005) Additionally, VE-cadherin is required to prevent disassembly of blood vessel walls (May, 2005; Hordijk, 1999; Crosby, 2005) and to coordinate the passage of macromolecules through the endothelium (Fukuhara, 2005; Kooistra, 2005). Tyrosine phosphorylation may provide the regulatory link, as increased phosphorylation of cadherins and potential dissociation of the cadherin/catenin complex results in decreased cell-cell adhesion and increased permeability (Ozawa, 1998; Potter, 2005).

Recent evidence has demonstrated that Rac1-induced reactive oxygen species (ROS) disrupt VE-cadherin based cell-cell adhesion (Van Wetering, 2002).

The mechanisms by which ROS affect endothelial permeability have not been fully characterized. VEGF has been reported to induce NADPH oxidase activity and induce the formation of ROS (Abid, 2000 & 2001).

In many cell types, including endothelial cells the major producer of ROS is NADPH oxidase (Babior, 2000).

These data indicate that VEGF treatment leads to the production of ROS and that these ROS are required for the increase in microvascular permeability.

Tyrosine phosphorylation of various adherens junction molecules in the endothelium is indicative of decreased junctional integrity (Volberg, 1992; Birchmeier, 1994). One of the major adhesion molecules of endothelial junctions is VE-cadherin. VEGF-induced

ROS production regulates the tyrosine phosphorylation of adherens junction proteins VE-cadherin, β-catenin, and p120-catenin and their association. VEGF has a well known and well studied role in angiogenesis. However, the molecular mechanisms that regulate VEGF-induced permeability in the microvasculature, the first described function of VEGF, remain incompletely understood (Senger, 1983; Senger, 1986). It was shown that VEGF regulates microvascular permeability through the activation of Rac1 and the production of ROS. These molecules, in turn, regulate the tyrosine phosphorylation of adherens junction proteins VEcadherin and β-catenin, ultimately regulating junctional integrity (Monaghan-Benson, 2009).

1.6. VEGF, IRON DEFICIENCY AND ANGIOGENESIS

Cellular iron deficiency increased HIF-1a, VEGF, and angiogenesis, suggesting that systemic iron deficiency might play an important part in the tumor angiogenesis. Iron is an essential metal for all living organisms participating in cellular processes, such as DNA synthesis, enzyme functions, and oxygen transport. Cellular iron metabolism is homologous among most cell types; cellular iron homeostasis is primarily mediated by transferrin (Tf), transferrin receptor-1 (TfR1), and ferritin (Pantopoulos, 2004; Rouault, 2006; Muckenthaler, 2008). Tf is an iron transport protein with two iron-binding sites and is mainly found in the bloodstream, from which it circulates and delivers iron throughout the body. TfR1 is a ubiquitous membrane protein forming a complex with Tf, which initiates membrane endocytosis and serves as a major cellular iron uptake pathway.

Excess iron within cells is stored in ferritin. When cells are iron deficient, iron regulatory proteins (IRPs) bind to iron responsive elements (IRE) in the 3′ untranslated region (UTR) of TfR1 mRNA as well as 5′ UTR of ferritin mRNA. The IRP

binding to 3′-UTR results in TfR1 upregulation and increased iron uptake, but its binding to 5′-UTR of ferritin leads to its downregulation and decreased iron storage (Rouault, 2006; Muckenthaler, 2008; Recalcati, 2010).

HIF-1a is a critical transcription factor for the regulation of vascular endothelial growth factor (VEGF), a potent inducer of tumor angiogenesis and metastasis (Bertout, 2008). However, direct evidence of iron deficiency on VEGF is lacking and the effect of iron deficiency on tumor angiogenesis has not yet been studied.

To further associate iron deficiency with HIF-1a, VEGF, and angiogenesis, using iron supplementation causes a destabilization of HIF-1a , a result which supports the idea that iron deficiency contributes to HIF-1a induction and stabilization. Further confirmation, the effects of iron supplementation also extended beyond cellular HIF stabilization and into downstream HIF signaling, which lead to a lower level of VEGF and a decline in the in vitro angiogenesis .

1.7. FUNCTION OF VEGF IN TUMORS

VEGF plays a crucial role in growth of most primary tumors and the subsequent process of metastasis. In many human tumors is observed an up-regulation of VEGF and its mRNA (mRNA and VEGF receptor), an increase in the levels of VEGF found both within the tumor into the circulation. Tumor growth requires vascularization that is mediated by VEGF. The VEGF released from the tumor acts at the level of VEGF receptors present on endothelial cells and activate angiogenesis. Thus, the VEGF stimulates the formation of a new and immature blood supply that has structural and functional abnormalities. The production of VEGF by the tumor creates a positive feedback by which the angiogenesis induced by VEGF allows further tumor growth.

As a result of this fast-growing cancer exceeds the limits of its blood supply and the contribution of oxygen and nutrients becomes inadequate, resulting in areas of hypoxia.

In a rapidly growing tumor, O2 demand increases and O2 delivery decreases, primarily because of: A) insufficient blood supply (at least to some tumor areas) and B) increasing diffusion distances between the blood vessels and the O2-consuming cells (Vaupel, 1989; Giordano, 2001). This leads to hypoxia in the expanding tumor mass, triggering events that stimulate angiogenesis in an effort to ameliorate the hypoxic condition. In tumor tissue, the ability to induce angiogenesis is associated with the development of an aggressive phenotype, as metastatic cells have more opportunity to enter the circulation in a well-vascularized tumor and thereby escape their hostile environment (Goonewardene, 2002; Hanahan, 1996). Production of VEGF is driven by hypoxia via transcription activation of the VEGF gene by HIF-1 (Forsythe, 1996). The basic importance of HIF-1 in the angiogenic process has been demonstrated in several experimental and clinical studies (Maxwell, 2002). Carmeliet et al. reported a reduced hypoxic induction of VEGF in vitro in mouse embryonic stem cells with inactivated HIF-1α genes (Carmeliet, 1998).

In vivo studies, found that HIF-1α–/– embryonic stem-cell-derived tumors had fewer blood vessels and impaired hemodynamics within the tumor mass, and further, that HIF-1α knockout mice died in utero with a complete lack of cephalic vasculature as a consequence of disrupted angiogenesis (Ryan, 1998 & 2000). Additionally, VEGF has been shown to stimulate migration of macrophages by activation of the VEGF receptor (Flt-1). Macrophages produce several angiogenic factors, including VEGF and tumor necrosis factor alpha (TNF-α) (Leibovich, 1987; Leek, 2000). At the clinical level, the results of the majority of over a dozen studies comprising more than 3,500 patients generally speak in favor of an independent prognostic impact of VEGF expression regarding

relapse-free and overall survival. Additionally, VEGF expression may be predictive of the anatomical site of first recurrence (Goonewardene, 2002).

In addition to VEGF, other angiogenesis-related gene products and receptors are regulated by HIF-1, including PDGF-B, VEGFR-1, endothelin-1, inducible nitric oxide synthetase (iNOS), monocyte chemotactic protein, adrenomedullin, and EGF. Several of these, including iNOS, endothelin-1, heme oxygenase 1, and adrenomedullin, have been shown to play roles in the regulation of local blood flow by the modulation of vascular tone (Wenger, 2002). Thus, it appears that HIF-1 not only mediates angiogenesis by VEGF induction but also influences tumor blood flow by more complex mechanisms involving target genes playing a role in vessel tone.

Yet another mechanism for stimulation of tumor angiogenesis is induction of HIF-1 and VEGF subsequent to somatic mutation. One example of this is seen in the loss of p53 tumor-suppressor activity either by direct mutational inactivation or by overexpression of mouse double minute, a ubiquitin protein ligase involved in the degradation of p53. Loss of p53 activity results in decreased hypoxia-mediated apoptosis, possibly increased HIF-1α expression (Ravi, 2000), and a subsequent increase in HIF-1-mediated transactivation of VEGF and other target genes, thereby facilitating tumor angiogenesis.

Angiogenesis, stimulated by VEGF, promotes the contact of tumor cells with blood vessels, providing a way for cells to invade the vessels themselves, Excessive production of VEGF leads to the formation of very permeable immature vessels, which will facilitate the invasion. VEGF is also involved in lymphangiogenesis. Stimulating the formation of giant lymphatic vessels, VEGF provides another possible route of metastatic spread. In humans, VEGF is overexpressed by a wide variety of different malignant tumours. Increased VEGF concentrations in the serum and plasma of patients indicate a poor prognosis for numerous tumor types and the magnitude of

VEGF concentration reflects tumour burden, response to therapy, and disease progression. Recently, VEGF has been shown to inhibit cells of dendritic cell lineage by impairing their antigen-presenting ability and limiting tumor cell detection by the immune system, thus enhancing metastasis.

1.8. VEGF IN CANINE PATIENTS

In dogs were identified similar VEGF isoforms described in humans differing due to the absence of a residues at the N-terminal region. In dogs, as in most other mammals, it should then use the following terminology: $VEGF_{164}$ and $VEGF_{120}$ (soluble isoforms) and $VEGF_{205}$, $VEGF_{144}$ and $VEGF_{188}$ (soluble isoforms, linked to the matrix extracellular). Between man and dog there is a complete homologue amino acid sequence involved in receptor development. This correspondence implies that the two proteins (human and canine) have the same binding properties and explains ability of canine $VEGF_{164}$ to activate human endothelial cells in same degree of $VEGF_{165}$; VEGFR-1 overexpression (together with that of VEGF) has been highlighted in several canine cancers (Scheidegger, 1999). Although VEGF expression has been documented in dogs with several solid tumors (Barbara kaser hotz) its role in canine malignancies is not well defined. In 2 recent studies (Wergin, 2004a & 2004b) concentrations of VEGF in dogs with naturally occurring neoplasia were found to be highest with malignant melanoma. These investigators also identified a significant difference among types of tumors and plasma VEGF concentrations (Wergin 2004b). High concentrations of VEGF were thought to be associated with the aggressiveness of the tumor but were not associated with tumor stage or tumor volume (Wergin 2004a). High concentrations of

VEGF were found in this study and were comparable to concentrations observed in the carcinoma category of tumors in the study (Wergin 2004a). VEGF serum concentration has been also investigated in healty and noneoplastic dogs and were found to be detectable in less than 10% of these patients whereas more than 40% of tumor dogs had detectable VEGF serum concentration (Troy, 2006). A recent study showed that canine patients with a serum VEGF level < 63 pg/mL had a doubled median overall survival if compared to those with a VEGF > 63 pg/mL (Marchetti, 2011).

1.8.1. VEGF IN MAST CELL TUMOURS (MCT)

Mast cell tumors (MCT) are frequent neoplasms in dogs (Macy, 1985; London, 2003; Misdorp, 2004). These tumours are often detected in the skin, but can also develop in and metastasize into other (visceral) organs (Macy, 1985; London and Seguin, 2003; Misdorp, 2004). The biological behaviour of canine mastocytomas varies considerably among patients (Rogers, 1996; Govier, 2003; London, 2003; Cahalane, 2004; Misdorp, 2004). Metastatic and progressive MCT usually exhibit a poor response to conventional antineoplastic drugs and thus have an unfavourable prognosis (Rogers, 1996; Govier, 2003; London, 2003; Cahalane, 2004; Misdorp, 2004). Aggressive MCT tumours usually exhibit a high grade histology (Patnaik, 1984). The expression of VEGF and VEGF receptors in neoplastic canine MC is poorly understood. Thus, whereas it is well known that normal mast cells in various species can express and release VEGF under certain circumstances (cell activation) and that mast cell-derived VEGF is involved in the regulation of wound healing and angiogenesis (Boesiger, 1998; Artuc, 1999; Szukiewicz, 2005), little is known so far about the expression of VEGF in neoplastic MC. Wimzal and colleagues have

recently shown that neoplastic human MCs in patients with systemic mastocytosis express detectable amounts of VEGF (Wimazal, 2002). Several studies have shown that normal mast cells can express and release VEGF, and that mast cell-derived VEGF is involved in the regulation of angiogenesis (Boesiger, 1998; Artuc, 1999; Szukiewicz, 2005). The expression of VEGF and of VEGF receptors in primary MCT in dogs and in canine mastocytoma cell line C2 has been investigated to determine whether VEGF acts as an autocrine growth regulator in neoplastic MCs (Rebuzzi, 2007) and although neoplastic cells often utilize VEGF as an autocrine growth factor (Giles, 2001; Gerber, 2003; Shinkaruk, 2003), in contrast to other neoplasms, mastocytoma cells may apparently do not utilize VEGF as an autocrine growth regulator (Rebuzzi, 2007). Only one study investigated in deep the relationship between cytosol and circulating VEGF levels, microvascular density (MVD) and mast cell density (MCD) in regulating tumour angiogenesis and progression of canine MCT (Patruno, 2009). It was noticed that serum VEGF may be an inaccurate indicator of circulating VEGF due to its release during sampling as no correlations between serum VEGF and MVD and between serum VEGF and malignancy grade of MCTs was found.

VEGF levels were found to be low in platelet poor plasma (P-PP) blood fraction suggesting that VEGF from serum samples is derived from platelets during the coagulation process as reported in human MCs (Verheul, 1997). Accordingly, VEGF levels in the P-PP could reflect the excess of circulating VEGF to the steady state with platelets levels without any biological clinical significance. VEGF levels from both cytosol and P-APR were higher than in P-PP and showed a statistically significant increase in G3 tumours when compared to G1 and G2. This study showed also that MCs of canine MCTs were the main source of circulating VEGF protein, which may be released by degranulation and then stored in platelets. In fact, was demonstrated that MCs in G3 MCTs subgroup contained few or no cytoplasmatic granules and this

parameter correlated with high MVD, high VEGF level from cytosol and high VEGF level from P-APR. On the contrary, MCs in G1 or G2 CMCTs were less degranulated, showing more methacromatic granules in their cytoplasm and these data correlated with low angiogenesis biblio.

2. OXIDATIVE STRESS AND ANTIOXIDANTS

2.1. OXIDATIVE STRESS AND ANTIOXIDANTS

In the last two decades there has been an explosive interest in the role of oxygen-free radicals, more generally known as “reactive oxygen species,” (ROS) and of “reactive nitrogen species” (RNS) in experimental and clinical medicine (Halliwell, 1999). ROS and RNS: (I) are generated during irradiation by UV light, by X-rays and by gamma rays; (II) are products of metal-catalyzed reactions; (III) are present as pollutants in the atmosphere; (IV) are produced by neutrophils and macrophages during inflammation; (V) are by-products of mitochondria-catalyzed electron transport reactions and other mechanisms (Cadenas, 1989). ROS/RNS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems (Valko, 2004). Beneficial effects of ROS involve physiological roles in cellular responses to anoxia, as for example in defence against infectious agents and in the function of a number of cellular signalling systems. One further beneficial example of ROS at low concentrations is the induction of a mitogenic response. In contrast, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids and membranes, proteins and nucleic acids (termed oxidative stress) (Poli, 2004). The harmful effects of ROS are balanced by the antioxidant action of non-enzymatic antioxidants in addition to antioxidant enzymes (Halliwell, 1996). Despite the presence of the cell’s antioxidant defence system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle, and radical-related damage to DNA, to

proteins and to lipids has been proposed to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions (Halliwell, 1999).

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons. The presence of unpaired electrons usually confers a considerable degree of reactivity upon a free radical. Those radicals derived from oxygen represent the most important class of such species generated in living systems (Valko, 2004). ROS can be produced from both endogenous and exogenous substances. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation (Inoue, 2003). Mitochondria have long been known to generate significant quantities of hydrogen peroxide. The hydrogen peroxide molecule does not contain an unpaired electron and thus is not a radical species. Under physiological conditions,

the production of hydrogen peroxide is estimated to account for about ~2% of the

total oxygen uptake by the organism. However, it is difficult to detect the occurrence of the superoxide radical in intact mitochondria, most probably in consequence of the presence of high SOD activity therein. Generation of the superoxide radical by mitochondria was first reported more than three decades ago (Loeschen, 1971). After the determination of the ratios of the mitochondrial generation of superoxide to that of hydrogen peroxide, the former was considered as the stoichiometric precursor for the latter. Ubisemiquinone has been proposed

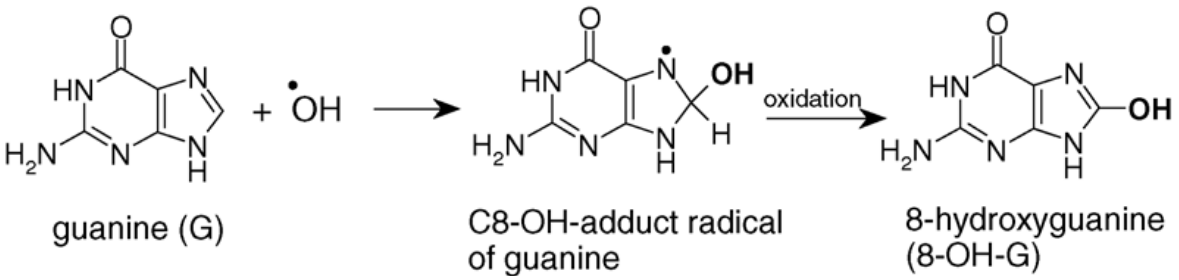
as the main reductant of oxygen in mitochondrial membranes (Inoue, 2003). Mitochondria generate approximately 2–3 nmol of superoxide/min per mg of protein, the ubiquitous presence of which indicates it to be the most important physiological source of this radical in living organisms (Inoue, 2003). Since mitochondria are the major site of free radical generation, they are highly enriched with antioxidants including GSH and enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which are present on both sides of their membranes in order to minimise oxidative stress in the organelle (Cadenas, 2000). Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified initially to hydrogen peroxide and then to water by Cu, Zn-SOD (SOD1, localised in the intermembrane space) and Mn-SOD (SOD2, localised in the matrix). Besides mitochondria, there are other cellular sources of superoxide radical, for example xanthine oxidase (XO), a highly versatile enzyme that is widely distributed among species (from bacteria to man) and within the various tissues of mammals (Li, 2002). Xanthine oxidase is an important source of oxygen-free radicals. It is a member of a group of enzymes known as molybdenum iron–sulphur flavin hydroxylases and catalyzes the hydroxylation of purines. In particular, XO catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion in the first step and hydrogen peroxide in the second (Valko, 2004). Additional endogenous sources of cellular reactive oxygen species are neutrophils, eosinophils and macrophages. Activated macrophages initiate an increase in oxygen uptake that gives rise to a variety of reactive oxygen species, including superoxide anion, nitric oxide and hydrogen peroxide (Conner, 1996). Cytochrome P450 has also been proposed as a source of reactive oxygen species. Through

the induction of cytochrome P450 enzymes, the possibility for the production of reactive oxygen species, in particular, superoxide anion and hydrogen peroxide, emerges following the breakdown or uncoupling of the P450 catalytic cycle. In addition, microsomes and peroxisomes are sources of ROS. Microsomes are responsible for the 80% H_2O_2 concentration produced in vivo at hyperoxia sites (Gupta, 1997). Peroxisomes are known to produce H_2O_2, but not $O_2^{\bullet-}$, under physiologic conditions. Although the liver is the primary organ where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these H_2O_2-generating mechanisms. Peroxisomal oxidation of fatty acids has recently been recognised as a potentially important source of H_2O_2 production as a result of prolonged starvation. The release of the biologically active molecules such as cytokines and others, from activated Kupffer cells (the resident macrophage of the liver) has been implicated in hepatotoxicological and hepatocarcinogenic events. Recent results indicate that there is a close link between products released form activated Kupffer cells and the tumour promotion stage of the carcinogenesis process (Klaunig, 2004). Reactive oxygen species can be produced by a host of exogenous processes. Environmental agents including non-genotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells. The induction of oxidative stress and damage has been observed following exposure to various xenobiotics. These involve chlorinated compounds, metal (redox and non-redox) ions, radiation and barbiturates. For example 2-butoxyethanol is known to produce ROS indirectly, which causes cancer in mice (Klaunig, 1997).

2.2. OXIDATIVE DAMAGE TO BIOMOLECULES

Reactive oxygen species are formed through a variety of events and pathways. It has been estimated that one human cell is exposed to approximately 1.5×10^5 oxidative hits a day from hydroxyl radicals and other such reactive species (Beckman, 1997). The hydroxyl radical is known to react with all components of the DNA molecule: damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Dizdaroglu, 2002). Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis and ageing. In fact, as is well established, in various cancer tissues free radical-mediated DNA damage has occurred. To date, more than 100 products have been identified from the oxidation of DNA. ROS-induced DNA damage involves single- or doublestranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Marnett, 2000; Cooke, 2003). The hydroxyl radical is able to add to double bonds of DNA bases at a second-order rate constant in the range of $(3–10)\times10^9 M^{-1}\ s^{-1}$ and it abstracts an H-atom from the methyl group of thymine and each of the five carbon atoms of 2' deoxyribose at a rate constant of approximately $2\times10^9 M^{-1}\ s^{-1}$ (Dizdaroglu, 2002). While OH-adduct radicals of DNA bases are generated via an addition reaction, the allylic radical derived from thymine and carbon-centred sugar radicals arise by abstraction reactions (Dizdaroglu, 2002). Further reactions of base and sugar radicals generate a

variety of modified bases and sugars, base-free sites, strand breaks and DNA–protein cross-links. The presence of 8-OH-G in human urine was first reported by Ames and co-workers (Shigenaga, 1989).



Shigenaga,1989

This oxidised DNA product is important because it is both relatively easily formed and is mutagenic and carcinogenic. It is a good biomarker of oxidative stress of an organism and a potential biomarker of carcinogenesis. We note that 8-hydroxyguanine undergoes keto-enol tautomerism and therefore 8-OH-G is often called 8-oxoguanine or 8-oxo-G, however, 8-oxo-G and 8-OH-G are equivalent. This base modification occurs in approximately one in 10^5 guanidine residues in a normal human cell. Ionising radiation, a carcinogenic and exogenous source of ROS, induced both urinary and leukocyte biomarkers of oxidative DNA damage (Halliwell, 1999). Tobacco smoking, another carcinogenic source of ROS, increases the oxidative DNA damage rate by 35–50%, as estimated from the urinary excretion of 8-oxo-G, and the level of 8-oxo-G in leukocytes by 20–50%. Measurements demonstrated that factors such as hard physical labour, day–night shift work, smoking and low meat intake significantly increased the 8-oxo-G level, while moderate physical exercise, such as sports reduced its level (Kasai, 2001). The potential connection between 8-nitroguanine and the process of carcinogenesis is unknown. In addition to the extensive studies devoted to the role

of oxidative nuclear DNA damage in neoplasia, there exists evidence about the involvement of the mitochondrial oxidative DNA damage in the carcinogenesis process (Inoue, 2003). Mutations and altered expression in mitochondrial genes encoding for complexes I, III, IV and V, and in the hypervariable regions of mitochondrial DNA, have been identified in various human cancers. The following points account for the fact that mitochondrial DNA is more susceptible to oxidation than nuclear DNA (Inoue, 2003): (I) under physiological conditions, the mitochondria convert ~5% of oxygen consumed into superoxide anion and subsequently hydrogen peroxide; (II) mitochondrial DNA repair capacity is limited, since they lack entirely the feature of nucleotide excision repair; (III) mitochondrial DNA is not protected by histones. Hydrogen peroxide and other reactive oxygen species have been implicated in the activation of nuclear genes that are involved in mitochondrial biogenesis, transcription, and replication of the mitochondrial genome. Although the region of tumour cells that possess mutated mitochondrial DNA and the extent to which mitochondrial DNA alterations participate in the cancer process have not been satisfactorily established, a significant amount of information supporting the involvement of the mitochondria in carcinogenesis exists (Penta, 2001). This connection supports the fact that fragments of mitochondrial DNA have been found to be inserted into nuclear DNA, suggesting a possible mechanism for activation of oncogenes. In conclusion, as observed with oxidative genomic DNA modification, oxidative damage and the induction of

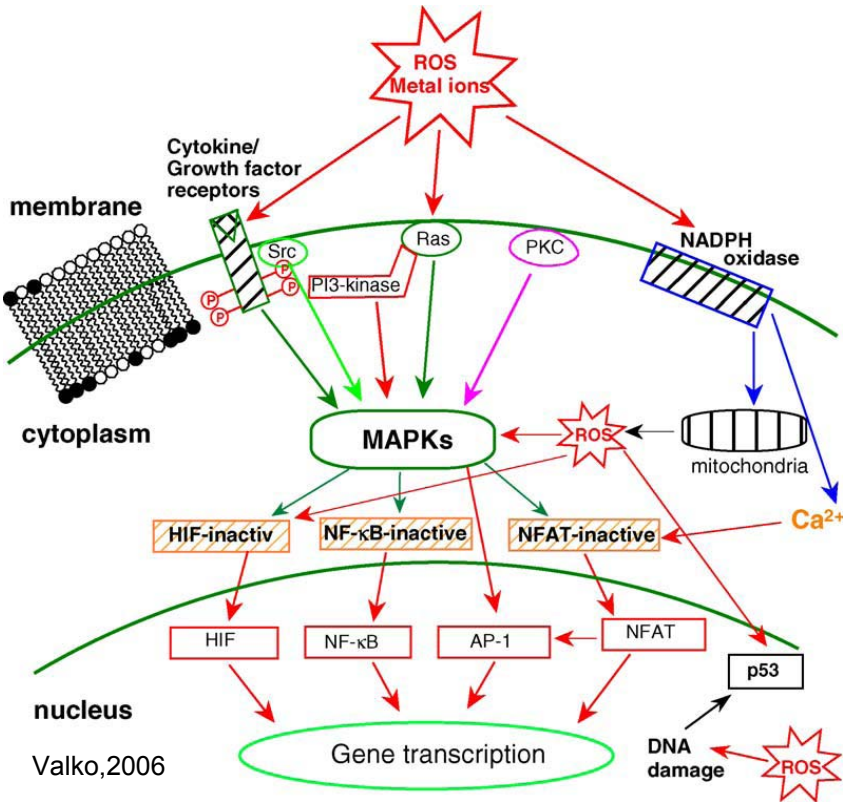
mutation in mitochondrial DNA may participate at multiple stages of the process of carcinogenesis, involving mitochondria-derived ROS, induction of mutations in mitochondrial genes, and possibly the insertion of mitochondrial genes into nuclear DNA (Penta, 2001). As described above, oxygen radicals may induce a number of DNA base alterations that can lead to mutagenesis. However, there are specific and general repair mechanisms that can repair DNA base modifications (Evans, 2004; Kasai, 2002). Off interest is the fact, that the efficiency of repair mechanisms may be enhanced following exposure to reactive oxygen species because expression of many DNA repair enzymes is upregulated following oxidative stress.

Since in nuclear DNA, ~90% of oxidised bases are repaired by single nucleotide repair mechanisms and the remaining 10% by long-patch base excision repair, the single nucleotide base excision repair is the primary pathway for repair of 8-OH-G. The first evidence of a repair mechanism for the 8-OH-G lesion was observed in irradiated mouse liver, where levels of this lesion were found to decrease with time (Kasai, 2002).

2.3. OXIDATIVE STRESS, CELL SIGNALLING AND CANCER

Cells communicate with each other and respond to extracellular stimuli through biological mechanisms called cell signalling or signal transduction (Poli, 2004; Thannickal, 2000). Signal transduction is a process enabling information to be transmitted from the outside of a cell to various functional elements inside the cell. Signal transduction is triggered by extracellular signals such as hormones, growth factors, cytokines and neurotransmitters (Hensley, 2000). Signals sent to the transcription machinery responsible for expression of certain genes are normally transmitted to the cell nucleus by a class of proteins called transcription factors. By binding to specific DNA sequences, these factors regulate the activity of RNA polymerase II. These signal transduction processes can induce various biological activities, such as muscle contraction, gene expression, cell growth and nerve transmission (Sah, 2000). While ROS are predominantly implicated in causing cell damage, they also play a major physiological role in several aspects of intracellular signalling and regulation (Palmer, 1997). It has been clearly demonstrated that ROS interfere with the expression of a number of genes and signal transduction pathways (Thannickal, 2000). Because ROS are oxidants by nature, they influence the redox status and may, according to their concentration, cause either a positive response (cell proliferation) or a negative cell response (growth arrest or cell death). As already mentioned above, while high concentrations of ROS cause cell death or even necrosis, the effects of ROS on cell proliferation occurred exclusively at low or transient concentrations of radicals. Low concentrations of superoxide radical and hydrogen peroxide in fact stimulate

proliferation and enhanced survival in a wide variety of cell types.ROS can thus play a very important physiological role as secondary messengers (Lowenstein, 1994). Other examples include regulation of the cytosolic calcium concentration (which itself regulates the above-mentioned biological activities), regulation of protein phosphorylation, and activation of certain transcription factors such as NF-kB and the AP-1 family factors (Storz, 2005).



ROS and metal ions primarily inhibit phosphorserine/threonine-, phosphotyrosine- and phospholipidphosphatases, most probably by interacting with sulphydryl groups on their cystein residues, which are oxidised to form either intramolecular or intermolecular disulphide bonds (Valko, 2004; Thannickal, 2000). These structural changes alter protein conformation which leads to the upregulation of several signalling cascades, most importantly growth factor kinase-, src/Abl kinase-, MAPK- and PI3-kinase-dependent signalling pathways. These signalling cascades

lead to the activation of several redox-regulated transcription factors (AP-1, NF-kB, p53, HIF-1, NFAT).

In veterinary oncology oxidant and antioxidant profile has not been evaluated so far, and few reports investigated mammary tumours (Szczubiał, 2004; Kumaraguruparan, 2005) and lymphomas (Vajdovich, 2005; Winter, 2009). In mammary tumours was observed an enhanced lipid peroxidation, attributed to overproduction of ROS, followed by an increase of the antioxidant barrier. Authors suggested that an increased lipid peroxidation and host antioxidant defences associated with the development of canine mammary tumours may offer a selective growth advantage to tumours cells over their surrounding normal counterparts.

In canine lymphoma patients a decrease in vitamin E secondary to lymphoma development was observed, suggesting an imbalance between ROS production and antioxidant protection. Glutathione Peroxidase (GSHPx) was found to be increased in one study (Winter, 2009) according to the fact that Glutathione is a principal cellular antioxidant and serves to regenerate oxidized vitamins C and E to their reduced and bioactive forms. On the other hand this is in contrast to the data reported by Vajdovich and collegues (Vajdovich, 2005) were GSHPx was found to be reduced, however this conflict could be related to differences in WHO classification and substage (including inclusion of more dogs with advanced disease in the study of Winter and collegues) or different immunophenotypes.

2.3.1. CYTOKINES AND GROWTH FACTORS SIGNALLING

A variety of cytokines and growth factors that bind to receptors of different classes have been reported to generate ROS in nonphagocytic cells. Growth factor receptors are tyrosine kinases (RTKs) that play a key role in the transmission of information from outside the cell into the cytoplasm and the nucleus (Neufeld, 1999). The information is transmitted via the activation of mitogen-activated protein kinases (MAPKs) signalling pathways (Hazzalin, 2002). ROS production as a result of activated growth factor receptor signalling includes epidermal growth factor (EGF) receptor (Bae, 1997), platelet-derived growth factor (PDGF) receptor (Catarzi, 2002), vascular endothelial growth factor (VEGF) (Neufeld, 1999). Further examples involve cytokine receptors (TNF-a and IFN-y) or interleukin receptors (IL-1b) (Sundaresan, 1996). Cytokines receptors fall into a large and heterogenous group of receptors that lack intrinsic kinase activity and are most directly linked to ion channels or G proteins. Cytokines such as TNF-a, IL-1 and interferon (IFN-y) were among those first reported to generate ROS in nonphagocytic cells (Chapple, 1997). It is generally accepted that ROS generated by these ligand/receptor-initiated pathways can function as true second messengers and mediate important cellular functions such as proliferation and programmed cell death. Abnormalities in growth factor receptor functioning are closely associated with the development of many cancers (Drevs, 2003). Several growth factor receptors (EGF, PDGF, VEGF) are affected by carcinogenic metals such as nickel, arsenic, cobalt and beryllium (Simeonova, 2002). The EGF receptor is associated with cell proliferation in normal cells. Nickel has been found to increase expression of the EGF receptors and overexpression of the EGF receptor has been observed in lung and urinary

cancers (Kim, 2001). Exogenous oxidative stress appears to stimulated secretion of heparin-binding EGF. VEGF is involved in proliferation and angiogenesis and also is induced by carcinogenic metals (Co, Ni and As) and hypoxia. Arsenic-induced VEGF expression appears to be associated with p38 (Leonard, 2004). Activation of both EGF and VEGF results in increases in cellular Ca(II). A similar effect was observed in a various cell types following treatment with Ni(II), Cd(II) and Be(II) compounds. The VEGF is probably most strongly activated by hydrogen peroxide. The PDGF is found in endothelial cells, fibroblasts and mesenchymal cells; the overexpression of PDGF has been found in lung and prostate cancers.

2.4. ANTIOXIDANT DEFENCE MECHANISMS IN CARCINOGENESIS

The effect of reactive oxygen and nitrogen species is balanced by the antioxidant action of non-enzymatic antioxidants, as well as by antioxidant enzymes. Such antioxidant defences are extremely important as they represent the direct removal of free radicals (pro-oxidants), thus providing maximal protection for biological sites. A good antioxidant should: (I) specifically quench free radicals; (II) chelate redox metals; (III) interact with (regenerate) other antioxidants within the "antioxidant network"; (IV) have a positive effect on gene expression; (V) be readily absorbed; (VI) have a concentration in tissues and biofluids at a physiologically relevant level; (VII) work in both the aqueous and/or membrane domains. The most efficient enzymatic antioxidants involve superoxide dismutase, catalase and glutathione peroxidase (Mates, 1999). Non-enzymatic antioxidants

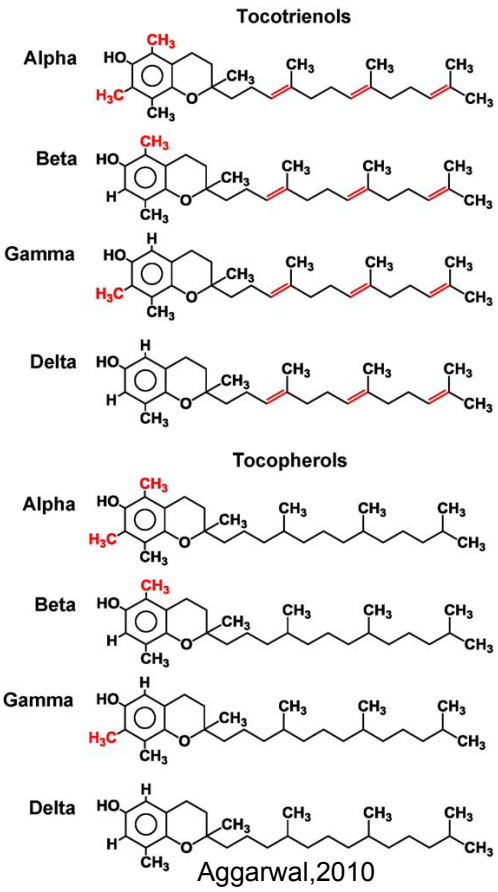
involve Vitamin C, Vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), natural flavonoids, a hormonal product of the pineal gland, melatonin and other compounds (McCall, 1999). Some antioxidants act in a hydrophilic environment, others in a hydrophobic environment, and some act in both environments of the cell. For example, Vitamin C reacts with superoxide in the aqueous phase while Vitamin E does so in the lipophilic phase. In contrast, a-lipoic acid is both water and fat soluble and therefore can operate both in cellular membranes and in cytosol. Certain antioxidants are able to regenerate other antioxidants and thus restore their original function. This process is called an "antioxidant network" (Sies, 2005). The redox cycles of vitamins E and C form such an antioxidant network. The capacity to regenerate one antioxidant by another is driven by the redox potentials of the [Red/Ox] couple. There is a link between increased levels of ROS and disturbed activities of enzymatic and non-enzymatic antioxidants in tumour cells.

2.4.1. VITAMIN E

Vitamin E is a fat-soluble vitamin that exists in eight different forms. α-Tocopherol is the most active form of vitamin E in humans and is a powerful biological antioxidant which is considered to be the major membranebound antioxidant employed by the cell (eight chemically distinct analogues are now known, consisting of alpha (α), beta (β), gamma (γ) and delta (δ)-tocopherols (TP) and alpha, beta, gamma and delta-tocotrienols (T3); all of themare referred to as

vitamin E. The tocopherols are saturated forms of vitamin E, whereas the tocotrienols are unsaturated and possess an isoprenoid side chain. (Burton, 1989).

Its main antioxidant function is protection against lipid peroxidation (ascorbic acid is regarded as the major aqueous phase antioxidant) (Prior, 2000). Recent evidence suggests that α-tocopherol and ascorbic acid function together in a cyclic-type of process. During the antioxidant reaction, α-tocopherol is converted to α-tocopherol radical by the donation of labile hydrogen to a lipid or lipid peroxyl radical. The α-tocopherol radical can thus be reduced to the original α-tocopherol form by ascorbic acid (Kojo, 2004). Several epidemiological trials reported the effect of the intake of Vitamin E supplements. It has been demonstrated that the intake of Vitamin E [200 IU (international units)/day] reduced the incidence of coloretal cancer by triggered apoptosis of cancer cells by inducing p21wafi/cip1, a powerful cell cycle inhibitor (White, 1997). Generally, the protective effect of Vitamin E is a result of the inhibition of free radical formation and activation of endonucleases. Other study reported negative results for Vitamin E in combination with Vitamin C and betacarotene to prevent colorectal cancer adenoma over a period of 4 years (Greenberg, 1994). Since Vitamin C regenerates Vitamin E, it has been proposed that addition of Vitamin E hinders the protective effect of Vitamin C against oxidative damage (Dreher, 1996).



Aggarwal,2010

Of great surprise was a recent trial which revealed that daily Vitamin E doses of 400 IU or more can increase the risk of death and should be avoided (Miller, 2005). According to the analysis, there is no increased risk of death with a dose of 200 IU per day or less, and there may even be some benefit.

2.4.1.1. VITAMIN E IN ANIMAL MODELS

In animals Tocotrienols exhibit activity in different models of both prevention and treatment of cancer. Perhaps the first report about the therapeutic potential of tocotrienols for cancer in animal models was by Kato et al., who in 1985 showed that tumor-bearing rats administered with tocotrienols had an extended life span (Kato, 1985). Komiyama et al. observed antitumor activity when tocotrienols were administered intraperitoneally to mice with established murine Meth A fibrosarcoma. They showed that tocotrienols were more effective than α-tocopherol, and among the tocotrienols, γ-tocotrienol was more effective than α-tocotrienol as an antitumor agent (Komiyama, 1989). They also showed that tocotrienols are better antioxidants than tocopherols. The growth of highly metastatic B16 melanoma in female mice was inhibited by tocotrienols, and δ-tocotrienol was more active than γ-tocotrienol in this setting (HE, 1997). In mice implanted with hepatoma, both γ-tocotrienol and δ-tocotrienol delayed tumor growth, and when examined for levels of tocotrienols, the tumors contained a specific accumulation of these analogues (Hiura, 2009). The antitumor effects of tocotrienols appear to be mediated in part through their ability to suppress

angiogenesis (Nakagawa, 2007). Suppression of angiogenesis is mediated through reduction in serum levels of VEGF and inhibition of the PI3K–AKT pathway. The inhibition of HMG-CoA reductase and the consequent decrease in serum cholesterol level has been linked with the tumor-suppressive action of tocotrienols (Elson, 1995). Tocotrienols have also been shown to enhance the antitumor effects of other agents. In one study, δ-tocotrienol was reported to enhance the growth-suppressive effects of lovastatin in the B16 melanoma model in mice (McAnally, 2007). Γ-Tocotrienol preferentially sensitized human prostate cancer in nude mice to radiation (Kumar, 2006). Besides antitumor effects against established tumors, tocotrienols have also been shown to be effective in cancer prevention models. Sundram et al. showed that palm oil, one of the richest dietary sources of tocotrienols, is effective in preventing 7,12-dimethylbenz[α]anthracene (DMBA)-induced mammary carcinogenesis in rats, but corn oil and soybean oil, which contain tocopherols but not tocotrienols, lack this activity (Sundram, 1989). Gould et al. reported a statistically significant increase in tumor latency in the DMBA-induced rat mammary tumor model with tocotrienols but not with tocopherols (Gould, 1995). Inhibition of tumor promotion by various palm-oil tocotrienols was also reported by Goh et al. (Goh, 1994), in an in vitro assay utilizing the activation of Epstein–Barr virus (EBV) early antigen expression in EBV-genome-carrying human lymphoblastoid cells. They showed that gamma- and delta-tocotrienol derived from palm oil exhibit strong activity against tumor promotion by inhibiting EBV early antigen expression in Raji cells induced by phorbol ester. However, alpha- and gamma-tocopherol and dimers of gamma-tocotrienol or gamma-tocopherol lack this activity (Khanna, 2006). Iqbal et al. showed that feeding tocotrienol-rich fraction (TRF; 10 mg/kg) to DMBA-administered rats suppressed

mammary carcinogenesis, and this correlated with declines in serum cholesterol, low-density lipoprotein (LDL)-cholesterol, and HMG-CoA reductase protein (Parker, 1993). Wada et al. examined the effect of 0.05% oral tocotrienols on spontaneous liver carcinogenesis in male mice and on glycerol-induced lung tumor promotion in male mice initiated with 4-nitroquinolone 1-oxide (Wada, 2005). Incidence of liver and lung tumors was almost 80% lower in treated animals than in untreated animals. Tocotrienols have been shown to prevent chemical-induced carcinogenesis of the liver (Iqbal, 2004) and found to suppress 2-acetylaminofluorene (AAF)-induced hepatocarcinogenesis (Ngah, 1991). In another study, Rahmat et al. (Rahmat, 1993) examined the effect of long-term administration of tocotrienols on hepatocarcinogenesis in rats. Liver carcinogenesis was induced by diethylnitrosamine and AAF in rats fed a diet containing 30mg/kg tocotrienols for 9 months. Expression ofbiomarkers of liver carcinogenesis suchas glutathione, alkaline phosphatase, and gamma-glutamyl transpeptidase was enhanced by the carcinogens but attenuated by tocotrienols, decreasing the impact of the carcinogens. A similar study by others confirmed these findings (Shamaan, 1993). All these studies suggest that tocotrienols have potential to both prevent and treat cancer.

2.4.1.2. VITAMIN E & MAST CELLS

Dietary vitamin E supplementation is reported to alleviate the symptoms of inflammatory diseases (Meydani, 1995). The pathogenesis of atopic dermatitis (AD), an immunoglobulin (Ig)E-mediated hypersensitivity reaction, is characterized by the release of inflammatory mediators from mast cells. Effects of the preformed mast cell mediator histamine are mediated through histamine receptors (H_1, H_2). Stimulation of H_1 on nerve cells causes pruritus. However, stimulation of H_2 on neutrophils, lymphocytes, and monocytes inhibits the release of inflammatory mediators. The effect of histamine on blood vessels seems to be mediated via H_1 and H_2, whereby it influences the vascular tone and permeability (Scott, 1995). It was hypothesized that in AD mast cells have an enhanced releasability for histamine (Hill, 2001). Proteases such as chymase and tryptase are also preformed mast cell mediators. In AD they are involved in the generation of bioactive peptides and they take part in the destruction of the extracellular matrix. The expression of mast cell proteases seems to be dependent on the microenvironment. Ricarelli and colleagues (Ricarelli, 1999) showed an inhibition of protease expression by vitamin E. Other mast cell mediators, such as prostaglandins, are produced and released immediately after stimulation. These *de novo* synthesized prostaglandins, especially prostaglandin D_2 (PGD_2), are known as pro-inflammatory mediators. PGD_2 leads to peripheral vasodilatation, inhibits platelet aggregation and functions as a chemo-attractant for neutrophils. The rate-limiting enzyme in prostaglandin production (prostaglandin H synthase, PGHS) is regulated by reactive oxygen species (ROS) (Chappel, 1997). The production and release of these mast cell mediators therefore seems to be

dependent on membrane composition and redox state (Wolfreys, 1997). Vitamin E accumulates in biological membranes and stabilizes them. Furthermore, mast cells are able to generate ROS in response to different stimulants. It has been shown that ROS generation induced during mast cell activation is prevented by the antioxidant diphenyleneiodinium leading to reduced release of histamine (Matsui, 2000). Gueck and colleagues demonstrate that vitamin E as a natural antioxidant also is involved in modulation of histamine release, prostaglandin D production and possibly chymase activity in the well-characterized canine mastocytoma cell line (C2). The author observed that the percentage of spontaneous and mastoparan (mast cell-degranulating peptide)-stimulated histamine release was reduced significantly in RRR-α-tocopherol-treated cells, otherwise chymase activity tended to be decreased by vitamin E but not tryptase (Gueck, 2002).

The effects of tocopherols on the proliferation and signaling pathways in mast cells was evaluateded on the human mastocytoma cell line (HMC-1) by Kempna and colleagues (Kempna, 2004) . The tocopherols inhibited HMC-1 cell proliferation with different potency ($\delta > \alpha = \gamma > \beta$). Growth inhibition correlated with the reduction of PKB (protein kinase B) phosphorylation by the different tocopherols. The reduction of PKB phosphorylation led to a decrease of its activity, as judged from a parallel reduction of GSK phosphorylation. The tocopherols did not significantly change oxidative stress in HMC-1 cells, suggesting that the observed effects were not the result of a general reduction of oxidative stress. Thus, the tocopherols interfered with PKB phosphorylation and reduced the proliferation of HMC-1 cells, possibly by modulating either phosphatidylinositol 3-kinase, a kinase phosphorylating PKB (PDK1/2), or a phosphatase that dephosphorylates it. Inhibition of proliferation and PKB signalling in HMC-1 cells by vitamin E suggested a role in preventing

diseases with mast cell involvement, such as allergies, atherosclerosis, and tumorigenesis (Reiter, 2007).

3 Iron profile

3.1. Iron

Iron is an essential element for many life forms, such as microbes, plants and higher animals. Its capacity to donate electrons and to receive plays a key role in many processes. This micronutrient is a cofactor of several systems redox enzyme, such as cytochromes; is involved in the transportation and storage of oxygen, because in the heme group of hemoglobin and myoglobin; takes part in DNA synthesis together with the enzyme ribonucleotidereduttase. However, the oxide-reducing capacity can result in the formation of radicals free that can damage cellular structures through the reactions of Fenton and Haber- Weiss (Gomme and McCann, 2005) The reaction leads to the formation of superoxide with the chemical formula O_2^-. Superoxide is biologically quite toxic and is deployed by the immune system to kill invading microorganisms. In phagocytes, superoxide is produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens. Mutations in the gene coding for the NADPH oxidase cause an immunodeficiency syndrome called chronic granulomatous disease, characterized by extreme susceptibility to infection, especially catalase positive organisms. In turn, micro-organisms genetically engineered to lack superoxide dismutase (SOD), lose virulence. Superoxide is also deleteriously produced as a byproduct of mitochondrial respiration , as well as several other enzymes, for example xanthine oxidase (Muller, 2007). Because superoxide is toxic, nearly all organisms living in the

presence of oxygen contain isoforms of the superoxide scavenging enzyme, superoxide dismutase, or SOD. SOD is an extremely efficient enzyme; it catalyzes the neutralization of superoxide nearly as fast as the two can diffuse together spontaneously in solution. Other proteins, which can be both oxidized and reduced by superoxide, have weak SOD-like activity (e.g. hemoglobin). Genetic inactivation ("knockout") of SOD produces deleterious phenotypes in organisms ranging from bacteria to mice and have provided important clues as to the mechanisms of toxicity of superoxide in vivo. The biological toxicity of superoxide is due to its ability to inactivate the group iron-sulfur enzymes (which are important in many metabolic processes), thereby releasing iron in the simple cell, according to the Fenton reaction and generate the radical hydroxyl which is highly reactive. In its form HO_2, superoxide can also initiate lipid peroxidation of polyunsaturated fatty acids. It also reacts with carbonyl compounds and halogenated carbons to create toxic peroxyl radicals. Superoxide may also react with nitric oxide (NO) forming $ONOO^-$. Due to this reason, superoxide is a cause of oxidative stress (Sawyer, 2001; Muller, 2007).

In mammals, there is no specific system for iron excretion, since its elimination is through the small quantities contained in the sweat (practically non-existent in domestic carnivores) or through the normal turnover of epithelial coating. The provisions martial contained in the enterocytes or in the keratinocytes in desquamation are, in fact, lost with the normal replacement of these. The body iron levels are then set only during the absorption phase in the proximal small intestine. The evolutionary pressure has selected an excellent system control uptake of this element, which is guaranteed with a normal amount of iron.

Especially in human medicine, the martial metabolism has long been subject of much research, but in recent years has only been possible to include with sufficient detail both the process of intestinal absorption of iron and its homeostatic regulation (Frazer and Anderson, 2005).

3.2. IRON HOMEOSTASIS

Six compartments can be distinguished in the distribution of body iron (Furlanello, 1999):

- Hemoglobin is the largest compartment of iron (approximately 67%).

- Ferritin and hemosiderin are the storage compartments (27%). Ferritin is a ubiquitous molecule, composed by thousands of iron atoms incorporated into a protein shell (apoferritin); Hemosiderin is the name given to the martial reserves arising from the catabolism of ferritin, which are contained in monocytes,-macrophages, bone marrow, spleen and liver's Kupffer cells.

- Myoglobin (3.5%), structurally similar to hemoglobin, is present in striated muscle fibers and heart muscle cells

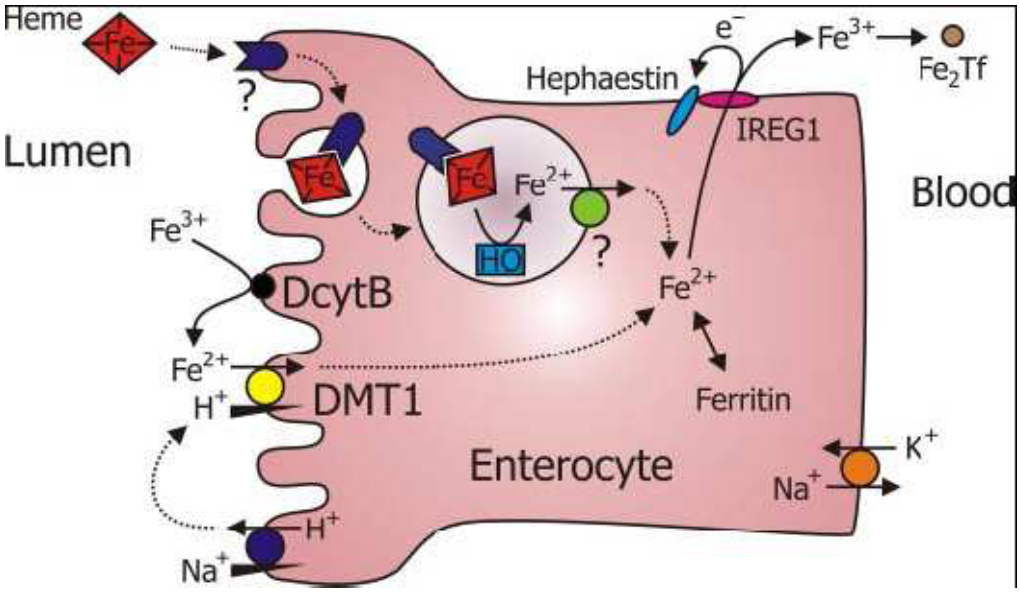
- The fleeting compartment is the iron in interstitial fluid and intracellular (approximately 2.2%); here can be attached to cell membranes or intracellular proteins, for a relatively short period, before being incorporated into the heme or in storage proteins.

- The tissue compartment of the iron is very low (0.2%) and includes many classes of enzymes, such as those of cytochrome

- The compartment plasma is the smallest, equal to about 0.08%, but is the most dynamic, because it is subject to changes (at least ten times a day). Is represented by transferrin (Tf), a glycoprotein, synthesized by liver, capable of carrying one or two atoms of ferric iron ($Fe3^{+}$).

3.3. IRON METABOLISM

The heme group consists of the complex Fe^{2} + -protoporphyrin IX and is associated with several classes of proteins, including those used for the transportation (eg hemoglobin) and storage (eg myoglobin) oxygen; it is also present in some enzymes which became part of prosthetic groups (eg tryptophan pirrolasi). The iron heme-conjugate is then contained in high concentrations in the diets of meat (Wells and Award, 1995). The heme non-conjugated iron is often called "iron ion" or "iron inorganic, although the latter is a misnomer because it is found, in the form of ferric and ferrous, bound to organic acids (eg citrate) or peptides (ferritin and albumin) (Frazer, 2005; Mackenzie, 2005).In dogs, the heme iron is absorbed directly into the market share of 30%, while the non-heme iron, most unfortunately, is poorly absorbed (less than 10%) (Furlanello, 1999).



Mackenzie e Garrick, 2005, modificato

Iron is quantitatively the most important metal of human metabolism. It is used for the transport of oxygen, as a vehicle of electrons and as a catalyst for biochemical reactions essential for growth and proliferation cell. Physiologically, iron is almost present as ferrous ion (Fe^{2+}) or ferric (Fe^{3+}). Due to these ions are highly reactive; iron is normally bound to transport proteins (transferrin), storage proteins (ferritin) or component of functional complex (iron-porphyrins, metallo-enzymes). The protein binding prevents the ionized iron intervenes in reactions that can damage cellular structures. Under physiological conditions, the amount of iron in the body is strictly controlled to avoid a progressive depletion of intracellular stores that can lead to iron deficiency anaemia and to accumulation of metal that exceeds the storage capacity of the organism. This event would produce toxic reactions in the cells which cause irreversible damage especially in the liver, pancreas and heart (iron overload) (Brittenham, 1994)

The erythroid bone marrow, erythrocytes of circulating blood or extravascular spaces (eg. of the spleen) together constitute a functional form called *eritrone*.

The *eritrone* includes most of the iron in the body and is the most important metabolic site. The main metabolic pathway consists of a unidirectional flow of iron from transferrin, the transport protein, towards the eritrone and from there to the monocyte-macrophage system and again to the transferrin plasmatica (Finch, 1982) An efficient recycling of iron, from red blood cells destroyed by macrophages to those newly formed in the bone marrow, is a characteristic of human metabolism martial. Physiologically, therefore, only a small part of total body iron is gained or lost every day.

The balance of iron is the result of the difference between the amount of iron absorbed and eliminated from the body. Since the human body is unable to actively eliminate iron, the balance of the metal is set, solely, by monitoring the absorption intestinal (Cook, 1987). The two major physiological factors that influence the absorption is the amount of iron in the deposits and the level of erythropoietic activity. If iron stores decrease, absorption increases (negative feedback). The absorption increases, also, with increased erythropoietic activity, both in physiological and pathological conditions, characterized by ineffective erythropoiesis. The main carrier of iron is intracorporeal transferrin produced by hepatocytes. The ferritin is storage of iron (up to 4500 iron atoms); 99% is intracellular, 1% free in serum. The other form of storage more stable is hemosiderin.

3.4. IRON AND CANCER

While iron plays an important role in many cellular functions, excess of iron storage induces DNA damage by generating hydroxyl radicals and thus promotes carcinogenesis. However, it remains unclear whether body iron levels that are commonly observed in a general population are related to oxidative DNA damage. It was examined the association between serum ferritin concentrations and levels of urinary 8-hydroxydeoxyguanosine (8-OHdG), a biomarker of systemic oxidative DNA damage and repair, in 528 Japanese men and women aged 21–67 years. Men had much higher ferritin levels than in women, and the levels were significantly greater in women aged 50 years or older than in women aged less than 50 years. Urinary 8-OHdG concentrations were significantly and positively associated with serum ferritin levels in all the subgroups. The Spearman rank correlation coefficients were 0.47, 0.76, and 0.73 for men overall, women aged less than 50 years, and women aged 50 years or older, respectively. These associations were materially unchanged after adjustment for potential confounding variables. In men, a more pronounced association was observed in non smokers than in smokers. These results suggest body iron storage is a strong determinant of levels of systemic oxidative DNA damage in a healthy population (Hori, 2009).

In experimental animals (Sawa, 1998), excess intake of heme iron induces the formation of radicals and the occurrence of colon cancer. In humans, high dietary intake of heme iron (Lee, 2005; Zhou, 2005) and blood measurements of iron (Hann, 1989; Knekt, 1994; Selby, 1988; Stevens, 1994) have been shown to be associated with an increased risk of cancer.

More recently, a randomized control trial found that phlebotomy, accompanied by a considerable reduction in serum ferritin levels, significantly decreased risk of cancer in men with a peripheral arterial disease (Zacharski, 2008). Such evidence suggests that cancer risk may vary according to body iron status even at levels commonly observed among the general population who do not have iron metabolic disorders. However, epidemiologic evidence regarding iron and cancer is far from consistent (Cross, 2006; Kato, 1999) and the finding from the above-mentioned trial should be interpreted cautiously because cancer was not the primary outcome (Zacharski, 2007). Investigations linking body iron to biomarkers of carcinogenesis may provide data to support or refute whether iron level currently admitted as normal influences cancer risk.

Urinary 8-hydroxydeoxyguanosine (8-OHdG) is a reliable biomarker of systemic oxidative DNA damage (Valko, 2006). Further, epidemiologic studies have shown that urinary 8-OHdG concentrations can predict cancer risk (Loft, 2006; Cooke, 2007; Thanan, 2008). However, few studies have been performed to quantify 8-OHdG levels in association with body iron status. Nakano et al. reported a positive correlation between serum ferritin concentrations and urinary 8-OHdG levels in 2507 healthy men and women (Nakano, 2003). However, they did not control for smoking or body mass index, factors known to be associated with 8-OHdG levels (Kasai, 2001; Mizoue, 2007). In a small study of 48 mild dyslipidemic men, Tuomainen et al. demonstrated a linear, positive relationship between serum ferritin and urinary 8-OHdG with adjustment for smoking, body mass index, and physical activity (Tuomainen, 2007). To further explore this issue, the present study examined the association between serum ferritin concentrations, a marker of body iron storage (Kohgo, 2008) and urinary 8-OHdG levels in healthy men and women while

adjusting for potential confounding factors. In conclusion, a strong positive association was found between urinary 8-OHdG levels and serum ferritin concentrations in Japanese workers.

This finding suggests that body iron storage is an important determinant of oxidative DNA damage, and thus supports a significant role for iron in carcinogenesis in a general population.

In people, hyperferritinemia, unrelated to iron stores, is recognized in several malignancies, including tumors of histiocytic origin (Yoda, 1980; Valberg, 1980; Esumi, 1989; Ya-You, 1998; Tang, 1987; Morita, 1981).

Hyperferritinemia has been documented in several malignancies of dogs, and particularly in istiocytic sarcoma (HS) and lymphoma (Newlands, 1994; Kazmierski, 2001). Ferritin is an iron storage and acute phase protein made by hepatocytes and cells of hematopoietic origin (Yoda, 1980). HS is an aggressive malignancy of antigen-presenting dendritic cells or phagocytic macrophages (Affolter, 2002; Moore, 2006) and a serum tumor marker specific for HS would be a valuable diagnostic aid. Friedrichs and collegues investigated the diagnostic utility of serum ferritin concentration as a tumor marker to differentiate dogs with HS from dogs with inflammatory disease, liver disease, IMHA (immune-mediated hemolitic anemia), and lymphoma. They found that hyperferritinemia is common in dogs with HS, otherwise the highest mean ferritin concentration was found in dogs with IMHA. The highest ferritin concentration in the HS category was observed in a dog with both marked hemolytic anemia and marked hepatic necrosis. Because dogs with hyperferritinemia could be found in all disease categories, ferritin concentration merely above the reference interval does not appear to be a useful discriminatory

test for HS, otherwise dogs with marked hyperferritinemia (≥7,200 ng/mL) were more likely to have HS or IMHA than any of the other diseases.

3.5. CAUSES OF IRON DEFICIENCY IN CANCER PATIENTS

Cellular iron metabolism is homologous among most cell types; cellular iron homeostasis is primarily mediated by transferrin (Tf), transferrin receptor-1 (TfR1), and ferritin (Pantopoulos, 2004; Rouault, 2006; Muckenthaler, 2008). Tf is an iron transport protein with two iron-binding sites and is mainly found in the bloodstream, from which it circulates and delivers iron throughout the body. TfR1 is a ubiquitous membrane protein forming a complex with Tf, which initiates membrane endocytosis and serves as a major cellular iron uptake pathway. Excess iron within cells is stored in ferritin. When cells are iron deficient, iron regulatory proteins (IRPs) bind to iron responsive elements (IRE) in the 3′ untranslated region (UTR) of TfR1 mRNA as well as 5′ UTR of ferritin mRNA. The IRP binding to 3′-UTR results in TfR1 upregulation and increased iron uptake, but its binding to 5′-UTR of ferritin leads to its downregulation and decreased iron storage (Rouault, 2006; Muckenthaler, 2008; Recalcati, 2010). Iron depletion in cells by iron chelator desferoxamine mesylate (DFO) has been shown to increase hypoxia inducible factor-1alpha (HIF-1a) (Dongiovanni 2008; Salnikow, 2004) HIF-1a is a critical transcription factor for the regulation of vascular endothelial growth factor (VEGF), a potent inducer of tumor angiogenesis and metastasis (Bertout, 2008). However, direct evidence of iron deficiency on VEGF is lacking and the effect of iron deficiency on tumor angiogenesis has not yet been studied. To provide proof of

the concept that iron deficiency plays a role in angiogenesis, used shRNA techniques to knockdown TfR1 levels in the triple negative breast cancer MDA-MB-231 cells. Because TfR1 is the gatekeeper of iron uptake, knocking down TfR1 is similar to closing the door for iron entry; it physically disables iron uptake and causes stable iron deficiency in cells, thus mimicking IDA. Their results showed that iron deficiency in cells increased the angiogenic potential of the cells.

This study is distinct from previous studies on iron overloadand its contribution to cancer initiation and promotion via oxidative stress pathways (Huang, 2003). In this study Has been demonstrated an important role for cellular iron deficiency in HIF-1a stabilization, VEGF formation, and angiogenesis, suggesting that systemic iron deficiency and anemia in young breast cancer patients may make them more susceptible to tumor recurrence.

Serum iron concentration can be considered potentially cancerogenic due to an increased formation of ROS and subsequent DNA damage and/or inactivation of proapoptotic factors as p53; on the other hand iron deficiency can be considered a tumour promoter, due to an increased HIF-1 production and subsequent increased VEGF serum concentrations.

The human or canine cancer patient may show many changes when compared to normal subjects.

- To have altered carbohydrate protein, and lipid metabolism.
- Deficiencies iron not only but also in zinc, chromium

Human cancer patients with these deficiencies exhibited well-recognized paraneoplastic syndromes such as decreased glucose tolerance, cachexia, and anaemia. These patients also failed to thrive during treatment of their neoplastic disease.

Iron metabolism is altered in the presence of inflammation or neoplasia. Serum concentrations of iron and total iron-binding capacity were lower in dogs with lymphoma and osteosarcoma compared to normal dogs. Serum ferritin concentration was higher in dogs with lymphoma compared to normal dogs and those with osteosarcoma. Signals for iron sequestration, as well as which protein principally serves as the iron storage pool, may differ within tumor type. Iron stores may be adequate in lymphoma and osteosarcoma, but the availability of iron for hemoglobin synthesis may be decreased. Such an effect could contribute to anemia of chronic disease in patients with inflammatory or neoplastic conditions. If sequestration is a protective mechanism against free iron oxidative injury, iron supplementation may be contraindicated. Alternatively, iron supplementation may improve erythropoiesis in clinically anemic patients, especially if the body is unable to release and use stored iron (Kathy J. 2001).

Zinc deficiency is a common finding in human cancer patients, especially in those with head and neck cancer. Cell-mediated immunity and natural killer (NK) cell activity are suboptimal in zinc-deficient patients. Zinc-deficient cancer patients experience increased treatment morbidity, treatment delays, and unplanned hospitalizations. Chromium deficiency is a causative factor in decreased glucose tolerance, a recognized paraneoplastic syndrome. It can lead to hyperinsulinemia and hyperlactatemia, both of which have been observed in human and animal cancer patients. Serum zinc concentrations were decreased in dogs with

lymphoma and osteosarcoma. The cause of decreased serum zinc concentration is unknown, but it may be due to inadequate dietary intake or absorption, or it may reflect redistribution to other tissues such as liver. Zinc is required for the activity of numerous metallo-enzymes, including those involved in wound healing and repair of cellular damage. Zinc also is important for regulation of immune activity, including optimizing the function of T-helper cells and NK cells. Zinc deficiency may adversely affect immune competence, predisposing affected individuals to development of cancer. Alternatively, zinc deficiency and its associated immune suppression may represent a paraneoplastic syndrome secondary to certain types of cancer. Serum zinc concentration in cancer patients may be an indicator of how well such patients will respond to treatment. Canine cancer patients may benefit from zinc supplementation during therapy, especially those with low serum concentrations or those suffering from immunosuppression. Dogs with lymphoma and osteosarcoma had lower serum chromium concentrations than normal dogs. Both human and animal cancer patients exhibit decreased glucose tolerance and decreased insulin sensitivity, resulting in hyperinsulinemia.

5. MATHERIAL AND METHODS

5.1 PATIENTS ELIGIBILITY

Canine patients with a histologically confirmed diagnosis of epithelial and mesenchimal tumours and histologically or cytologically confirmed diagnosis of cutaneous mast cell tumours were prospectively enrolled from 2008 to 2010. Post diagnosis staging work-up included a complete physical examination, haematology and serum biochemical profile, abdominal ultrasound and three standard view of the thorax were also performed. Staging work-up in patients with a confirmed diagnosis of mast cell tumour included also a fine needle biopsy of sentinel lymph node, liver and spleen. Two control groups of healthy and “non neoplastic disease” dogs were also designed. Client owned healthy dogs were judged to be normal if there were no health complaints reported by the owner and no abnormalities detected on physical examination. All tumour-bearing dogs and dogs with nonneoplastic diseases were client owned and referred to the Veterinary Teaching Hospital of Pisa University for evaluation of a variety of medical disorders. All the patients enrolled in the study had no previous medical treatment.

5.2 GROUPS DESIGN

Cancer patients were divided in three main groups:

- Mast cell tumours
- Epithelial tumours
- Mesenchimal tumours

Within the mast cell tumours' group, patients were divided in two sub-groups based on the clinical stage of the disease:

- Clinical stage 1 & 3: in this group were considered patients in clinical stage 1 and 3, when as stage 3 were classified multiple dermal lesions with no presence of metastatic disease. According to the World Health Organization clinical staging system for mast cell tumours (Owen, 1980).
- Clinical stage 2 & 4: in this group were considered patients in clinical stage 2 and 4 (metastatic disease), According to the World Health Organization clinical staging system for mast cell tumours (Owen, 1980).

Within the mast cell tumours' group, patients were divided in two sub-groups based survived and deceased:

- Survived: dogs alive at least 365 days after the diagnosis
- Deceased: dogs deceased due to causes tumour related

5.3 SAMPLES COLLECTION

Samples in EDTA and a serum gel tube were collected from canine cancer and nonneoplastic patients (12 hours fasted) and processed for haematology and complete serum biochemical profile at the time of the diagnosis (T_0); at T_0 thorax radiographs and abdominal ultrasound were also performed in all cancer patients. At T_0 two heparinised tube and two serum gel tube were also collected (from both cancer groups and control groups) and centrifuged within 1 hour after venipuncture at 3000 r.p.m for 5 minutes (refrigered centrifuge ALC PK 120R, ALC International S.r.l., Milano, Italy) and stored at -80°C. These extra tubes were used for VEGF, ROMs, BAP, VItamine E (α-tochopherol) and iron serum profile analyses.

5.4 LABORATORY ANALYSES

5.4.1. IRON PROFILE

iron status profile was evaluated on 500 µl of serum at the *Laboratorio d'Analisi Veterinarie San Marco"* (laboratorio d'analisi veterinarie San Marco, Padova, Italy) through:

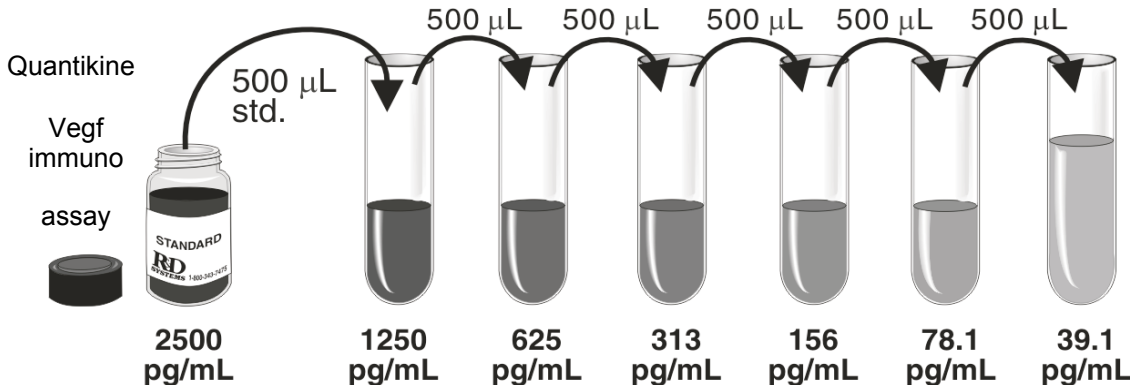
- Iron serum concentration* expressed as µg/dL
- Unsaturated iron-binding capacity (UIBC) serum concentration* expressed as µp/dL
- Ferritin serum concentration* expressed as ng/mL

- Serum derived iron parameters: Total iron-binding capacity [TIBC] expressed as µp/dL and saturation %

*laboratory methods are not shown on laboratory's request.

5.4.2. VEGF

VEGF serum concentration was evaluated using a monoclonal canine VEGF antibody commercial (sandwich enzyme immunoassay technique) kit (Quantikine canine VEGF immunoassay, R&D systems inc., Minneapolis, USA). Sample preparation consisted in the dilution of 150 µl of plasma with 150 µl of calibrator diluents RD6U. For reagents preparation 20 mL of Wash buffer concentrate were gently mixed into deionized or distilled water to prepare 500 mL of wash buffer. The substrate solution was prepared mixing the color reagents A and B together in equal volumes within 15 minutes of use and protected from light. The VEGF standard was reconstitute with 1.0 mL of calibrator diluent RD6U. Polypropylene tubes were disposed to produce a dilution series and 500 µl of calibrator diluent RD6U were added into each tube. The stock solution was used to produce a dilution series (below). Each tube was mixed thoroughly before the next transfer. The undiluted standard served as the high Standard (2500 pg/mL). Calibrator diluent RD6U serves as the zero standard (0 pg/mL).



100 µl of assay diluent RD1W and 100 µl of Standard were added to each plasma diluted sample and then covered with adhesive strip and incubate for 2 hours at room temperature. Each sample was aspirated and washed with 400 µl of wash buffer repeating the process twice for a total of three washes. 200 µl of canine VEGF Conjugate were added to each and sampled were covered with a new adhesive strip and Incubated for 2 hours at room temperature. Samles were washed again as described before and 200 µl of substrate solution were added to each sample and protected from light and incubated for 25 minutes at room temperature. After 50 µl of stop solution were added to each sample, optical density was determined within 30 minutes, using a microplate reader set to 450 nm. The results were expressed as pg/l

5.4.3. ROMS & BAP TEST

All the samples were examined with d-ROMs and BAP tests assessed by spectrophotometric method (Slim, SEAC, Florence, Italy). In d-ROMs test (Diacron International, Grosseto, Italy), reactive oxygen metabolites (hydroperoxides primarily) of a biological sample, in presence of iron released from plasma proteins by an acidic buffer, are able to generate alkoxyl and peroxyl radicals, according to

the Fenton's reaction. Such radicals, in turn, are able to oxidize an alkyl-substituted aromatic amine (N,N-dietylparaphenylendiamine), thus producing a pink-coloured derivative which is photometrically quantified at 505 nm (Alberti, 2000). The ROM's concentration runs directly parallel with colour intensity and is expressed as Carratelli Units (1 CARR U=0.08 mg hydrogen peroxide/dl). In BAP test (Diacron International, Grosseto, Italy) the addition of plasma sample to a coloured solution (thiocyanate), obtained by mixing a ferric chloride solution with a thiocyanate derivative solution, causes a decolouration, which intensity is measured photometrically at 505 nm and is proportional to the ability of plasma to reduce ferric ions (Benzie 1996). The results were expressed as µmol/l or reduced ferric ions.

ROMs analytic procedure:

After the preparation of the Standard solution (lyophilized), R_1 (chromogen) and R_2 (buffer) were brought to 37°C. Three solution were prepared: blank (R_1 + R_2 + H_20), sample (sample + R_1+R_2) and the calibrator (R_1+R_2+ calibrator). Solution were mixed and incubated at 37°C for 1 minute. Then the solutions underwent photometric reading (505 nm) at 37° for 1 minute. Further readings were performed at 1, 2 and 3 minutes. For the absorbance values obtained for the sample and the calibrator the absorbance of the blank reagent was subtracted. Results were expressed in U CARR according with the following equation: U CARR = ∆abs/min x F. ∆abs/min are the mean

differences of absorbance values measured at minute 1, 2 and 3. F is a correction factor with a predetermined value. Caratelli unite is equal to 0,08 mg di H_2O_2/dl.

BAP analytic procedure

R_1 (chromogen), R_2 (solution of ferric chloride [FECl3]) and R_3 calibrator were brought to 37°C. Three solution were prepared: blank (R_1 + R_2 + H_20), sample (sample + R_1+R_2) and the calibrator (R_1+R_2+ calibrator). Solution were mixed and incubated at 37°C for 5 minute. Then the solutions underwent photometric reading (505 nm) at 37° for 1 minute. Test's results, the iron-reducing antioxidant power of plasma, were expressed in μ iron-reducing antioxidant equivalents per liter of sample, according to the formula:

$$\frac{[\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}]}{[\text{Abs}_{\text{blank}} - \text{Abs}_{\text{calibrator}}]} \text{ X [calibrator]}$$

[Abs] are the absorbance values of the solutions measured at 505 nm

[calibrator] is the concentration of the calibrator expressed as μmol/l.

5.4.4. VITAMIN E (α-TOCHOPHEROL)

α-tochopherol was assessed through a HPLC system by using a Chromosystems-Diagnostic Kit HPLC & LC/MS® (Munchen, Germany). 200 μl of serum were added

to 20 μl of internal standard and to 25 μl of precipitation reagent I. Samples were vortex for 30 seconds and added with 400 μl of precipitation reagent II. Then samples were vortex for 30 seconds and centrifuged at 10000 rpm for 10 minutes. 50 μl of supernatant were submitted to HPLC analysis. The HPLC system consisted of a Series 200 Perkin Elmer gradient Pump (Norwalk, CT, USA) coupled to a Series 200 Perkin Elmer variable UV detector (Norwalk, CT, USA), which was set at 325 nm and 254 nm. HPLC was interfaced with a personal computer by using an interface SERIES 600 Perkin Elmer (Norwalk, CT, USA). Integration of pikes was performed through Turbochrome Navigator software (Perkin Elmer). A chromatographic column (CHROMOSYSTEMS®, Munchen, Germany) was used. Instruments showed a wave length of 325 nm, which was switched to 295 nm after 3.5 minutes. The flux was set at 1.5 ml/min and temperature of the column was 25°C. The results were expressed as μmol/l

CHROMSYSTEMS–DIAGNOSTICS BY HPLC & LCMS/MS® , München, Germany

5.5 STATISTICAL ANALYSIS

Data were tested for normality by the Anderson-Darling Normality test. Data not normally distributed were log 10 transformed before analysis, except for iron, ferritin, UIBC, TIBC, Saturation and VEGF. Hematology data, serum biochemistry data, BAP, ROMs and Vitamin E were evaluated with one way Anova and Tukey's Pairwise comparisons when differences between mast cell tumor, epithelial tumor, mesenchimal tumor, healthy patients and nonneoplastic patients. To analyze the difference within groups () the two samples t-test was used. For iron, ferritin, UIBC, TIBC, Saturation and VEGF that were not normally distributed, non-parametric tests were used (Mann-Whitney Test and Kruskal-Wallis Test). Correlations were evaluated Pearson's test. Data were expressed as means and standard deviations, except where differently defined. Analyses were performed using the statistical package Minitab 16.1.1 (Minitab Inc., State College, USA).

6. RESULTS

6.1 GROUPS DESCRIPTION

The study included 24 dogs in the *mast cell tumor group* (12 females, 5 neutered females and 7 males), 10 dogs in the *mesenchimal tumor* group (7 females and 3 males), 12 dogs in the *epithelial tumor* group (7 females, 1 neutered female and 4 males), 12 dogs in the *non neoplastic disease* control group (6 females and 4 males) and 10 dogs in the *healthy* control group (7 females and 3 males). Mean age in the groups were 8.3±2.1, 9.6±1.3, 10.5±1.9, 6.8±2.2 and 7.3±3.3 years, respectively.

***Mast cell tumors* group**				
Breed	**Sex**	**Age**	**Diagnosis**	**Clinical Stage**
Labrador	Female	10	MCT	2°b
Boxer	Female	7	MCT	3°a
Boxer	Female	8	MCT	1°a
French Bulldog	Male	5	MCT	1°a
Labrador	Male	10	MCT	1°a
Labrador	Female	9	MCT	3°b
Labrador	Female neutered	8	MCT	1°a
Cross breed	Male	10	MCT	3°a
Dobermann	Female neutered	10	MCT	1°a
Cross breed	Male	4	MCT	1°a
Boxer	Female neutered	7	MCT	4°b
Labrador	Female neutered	10	MCT	2°b
German Shepherd	Male	10	MCT	4°b
Beagle	Female	10	MCT	1°a
Golden retriever	Female neutered	4	MCT	3°a
Dachshund	Female	6	MCT	1°a
Labrador	Female	9,5	MCT	4°b
Cross breed	Male	8	MCT	4°b
Boxer	Female	9	MCT	1°a
Cross breed	Female	8	MCT	4°b
Cross breed	Female	7	MCT	1°a
Labrador	Female	8	MCT	1°b
Boxer	Female	11	MCT	1°a

***Mesenchimal tumors* group**				
Breed	**Sex**	**Age**	**Diagnosis**	**Clinical Stage**
Maremma Sheepdog	Female	9	Mixosarcoma	T4 N0 M0
Cross breed	Female	9	HSA	T2 N0 M0
Cross breed	Female	11	HSA	T2 N2 M1
Cross breed	Male	8	Condrosarcoma	T2 N0 M0
Cross breed	Female	11	Lipoblastoma	Unknown
Cross breed	Male	11	HSA	T2 N0 M0
Boxer	Male	10	HSA	T3 N2 M1
German Shepherd	Female	8	Liposarcoma	T4 N0 M0
Golden retriever	Female	8	Mixosarcoma	T4 N0 M0
Cross breed	Female	11	OSA	T2 M0 G2b

***Epithelial tumors* group**				
Breed	**Sex**	**Age**	**Diagnosis**	**Clinical Stage**
Beagle	Male	10	TCC	T2 N0 M0
Dobermann	Male	7,5	Melanoma (Skin)	T1 N0 M0
Cross breed	Female neutered	11	Mammary CA	T2 N0 M1
Irish Setter	Female	11.5	Melanoma (oral)	Stage I
Cross breed	Female	12	Undifferentiated CA	T2 N0 M0
Cross breed	Female	14	Anal sac CA	T2 N3 M0
Siberian Husky	Female	12	SCC (oral)	T4 N2 M0
Cross breed	Male	11	melanoma (oral)	Stage II
German Shepherd	Female	9	SCC (oral)	T4 N1 M0
Boxer	Female	9	Melanoma (skin)	T3 N1 M0
AMSTAF	Female	8	TCC	T2 N0 M0
Cocker spaniel	Male	11	Melanoma (oral)	Stage IV

***Non neoplastic diseases* control group**			
Breed	**Sex**	**Age**	**Diagnosis**
Cross breed	Female	10	GI tract disease
Cross breed	Male	12	Neurologic
Pincher	Female	2	LT respiratory disease
German Shepherd	Male	2	myositis
Golden retriever	Female	6	pyometra
Labrador	Male	9	GI tract disease
Cross breed	Female	8	IRC
Cross breed	Male	9	IRC
Cross breed	Female	6	IRC
Cross breed	Female	9	IRC

***Healthy* control group**		
Breed	**Sex**	**Age**
Cross breed	Female	10
Setter irlandese	Male	8
Pointer	Female	7
German Shepherd	Male	8
Breton	Female	5
labrador	Female	3
Cross breed	Female	6
Dobermann	Male	8
Cross breed	Female	4
German Shepherd	Female	9

6.2 IRON SERUM STATUS, SERUM VEGF AND OXIDATIVE STRESS PATTERN BETWEEN GROUPS

Iron serum concentration was significantly higher in mast cell tumors (*Mast cell tumors* group) compared to mesenchimal tumors (*Mesenchimal tumors* group) (146.5±45.6 µg/dL and 96.5±50.8 µg/dL, respectively), while ferritin was significantly lower in the healthy dogs compared to all other groups (Table 1). No statistical significant differences were observed in UIBC, TIBC and iron saturation % between groups.

Vascular Endothelial Growth Factor (VEGF) was significantly lower in healthy dogs (*Healthy* control group) compared to mast cell tumor, epithelial tumor and non neoplastic disease (*Non neoplastic diseases* control group). Patients affected by non neoplastic diseases showed a significantly higher VEGF than the mesenchimal tumors dogs (Table 2).

Reactive Oxygen Molecules (ROMs) were significantly higher in cancer patients groups (*Mast cell, Mesenchimal* and *Epithelial* groups) than both the control groups (*Healthy* and *Non neoplastic diseases* control groups). *Healthy* control patients showed significantly higher **Biological Antioxidant Potential** (BAP) and **Vitamin E** values compared to the *Non neoplastic diseases* group, and in the *Healthy* group BAP was also significantly higher than in the *Epithelial* and *Mast cell tumor* groups groups (Table 3).

Table 1 Mean, standard deviation and median values of iron, ferritin, UIBC, TIBC and saturation in the three oncologic patient groups (*Mast cell, Mesenchimal* and *Epithelial* groups) and in the two control patients groups (*Non neoplastic diseases* and *Healthy* control groups).

	N°	Iron	Ferritin	UIBC	TIBC	Saturation
		µg/dL	ng/mL	µp/dL	µp/dL	%
MCT	24					
mean		146.6	166.6	205.2	353.7	44.1
St. Dev.		45.6	101.7	68.2	74.8	15.5
median		145.5 [B]	129.5 [B]	210.5	355.0	41.1
Mesenchimal	10					
mean		96.5	192.4	217.1	316.7	31.0
St. Dev.		50.8	89.1	77.0	93.4	15.1
median		91.5 [A]	189.0 [B]	243.0	323.0	28.7
Epithelial	12					
mean		137.3	191.9	241.2	380.2	36.8
St. Dev.		53.6	106.2	74.6	80.9	12.5
median		114.5	210.5 [B]	259.0	384.0	33.5
Non neopl. dis. CTR	10					
mean		115.8	152.4	231.1	345.9	35.9
St. Dev.		26.2	60.2	63.9	75.0	8.8
median		102.5	146.5 [B]	251.0	372.0	34.5
Healthy CTR	10					
mean		120.9	89.0	230.8	358.6	33.5
St. Dev.		19.2	18.1	28.0	28.5	4.9
median		121.0	85.5 [A]	239.5	351.5	33.1
Kruskal-Wallis Test		P = 0.023	P = 0.020	n.s.	n.s.	n.s.

Mann-Whitney Test: A≠B, C≠D (P<0.05)

Table 2 Mean, standard dediation and median values of VEGF in the three oncologic patients groups (*Mast cell, Mesenchimal* and *Epithelial* groups) and in the two control patient groups (*Non neoplastic diseases* and *Healthy* control groups).

	n	VEGF
		pg/mL
MCT	24	
mean		99.6
St. Dev.		26.8
median		85.6[B]
Mesenchimal	10	
mean		91.3
St. Dev.		48.4
median		79.5 [C]
Epithelial	12	
mean		137.6
St. Dev.		135.4
median		99.3 [B]
Non neopl. dis. CTR	10	
mean		199.4
St. Dev.		165.8
median		129.8 [B,D]
Healthy CTR	10	
mean		76.3
St. Dev.		23.4
median		73.1 [A]
Kruskal-Wallis Test		P = 0.012

Mann-Whitney Test: A≠B; C≠D (P<0.05)

Table 3 Mean, standard deviation and median values of ROMs, BAP and Vitamin E in the three oncologic patients groups (*Mast cell, Mesenchimal* and *Epithelial* groups) and in the two control patient groups (*Non neoplastic diseases* and *Healthy* control groups).

	N°	ROMs	BAP	Vitamin E
MCT	23	U Carr	mmol/L	µmol/L
mean		136.7 [B]	2026.0 [B]	26.1 [A]
St. Dev.		33.0	220.7	11.8
median		139.8	1953.0	25.6
Mesenchimal	10			
mean		154.4 [B]	2217.1	17.0
St. Dev.		23.3	243.7	5.6
median		149.6	2238.5	15.4
Epithelial	12			
mean		155.4 [B]	2202.0 [B]	22.9
St. Dev.		25.8	240.2	9.9
median		150.9	2189.5	24.2
Non neopl. dis. CTR	10			
mean		96.3 [A]	2156.3 [B]	11.7 [B]
St. Dev.		41.7	875.5	12.7
median		91.5	1949.0	6.3
Healthy CTR	10			
mean		71.9 [A]	2762.1 [A]	26.3 [A]
St. Dev.		11.3	643.9	5.3
median		71	2553.5	23.8
One way ANOVA		P=0.000	P=0.003	P=0.001

Tukey's pairwise comparisons: A≠B (P<0.05)

6.3 DIFFERENCES IN IRON SERUM STATUS, SERUM VEGF AND OXIDATIVE STRESS PATTERN WITHIN MCT PATIENT GROUPS

Iron serum status (Table 4), VEGF serum concentration (Table 5), ROMs, BAP and Vitamin E (Table6) showed no statistically significant differences between the two MCT sub-groups based on the clinical stage of the disease (*Clinical stage 1 & 3*, *Clinical stage 2 & 4*)

Table 4 Mean, standard deviation and median values of iron, ferritin, UIBC, TIBC and saturation in *Clinical stage 1 & 3* MCT and *Clinical stage 2 & 4* MCT

	N°	Iron	Ferritin	UIBC	TIBC	Saturation
Clinical stage 1 & 3	17	µg/dL	ng/mL	µp/dL	µp/dL	%
mean		138.4	145.2	214.6	353.2	42.3
St. Dev.		21.8	83.6	65.4	69.1	11.8
median		143.0	127.0	210.0	370.0	40.5
Clinical stage 2 & 4	7					
mean		166.3	218.4	182.4	354.9	48.7
St. Dev.		77.9	128.8	74.6	93.3	22.8
median		148.0	224.0	211.0	328.0	41.4
Mann-Whitney test		n.s	n.s	n.s	n.s	n.s

Tukey's pairwise comparisons: A≠B (P<0.05)

Table 5 Mean, standard deviation and median values of VEGF in *Clinical stage 1 & 3* MCT and *Clinical stage 2 & 4* MCT

	N°	VEGF
Clinical stage 1 & 3	17	pg/mL
mean		98.3
St. Dev.		28.5
median		84.5
Clinical stage 2 & 4	7	
mean		103.0
St. Dev.		23.8
median		85.9
Mann-Whitney test		n.s

Table 6 Mean, standard deviation and median values of ROMs, BAP and Vitamin E in *Clinical stage 1 & 3* MCT and *Clinical stage 2 & 4* MCT

	N°	ROMs	BAP	Vitamin E
Clinical stage 1 & 3	16	U Carr	mmol/L	µmol/L
mean		138.6	2001.9	25.9
St. Dev.		30.7	163.5	12.9
median		147.5	1952.0	23.6
Clinical stage 2 & 4	7			
mean		132.5	2081.0	29.5
St. Dev.		40.1	326.7	9.3
median		119.4	2104.0	32.6
Two samples t-test		n.s.	n.s.	n.s.

Iron serum status (Table 7), VEGF serum concentration (Table 8), ROMs, BAP and Vitamin E (Table 9) showed no statistically significant differences between the two MCT sub-groups based on survived and deceased patients (*survived* and *deceased*)

Table 7 Mean, standard deviation and median values of iron, ferritin, UIBC, TIBC and saturation in *Deceased* MCT and *Survived* MCT.

	N°	Iron	Ferritn	UIBC	TIBC	Saturation
Deceased	5	µg/dL	ng/mL	µp/dL	µp/dL	%
mean		164.8	277.0	170.4	335.2	49.1
St. Dev.		94.0	99.3	87.9	92.2	27.6
median		138	254	200	320	40.8
Survived	18					
mean		141.4	140.8	214.6	360.2	42.9
St. Dev.		24.7	83.2	63.4	73.4	11.8
median		146.2	124.5	211.5	370	41.4
Mann-Whitney test		N.S.	P=0.0101	N.S.	N.S.	N.S.

Table 8 Mean, standard deviation and median values of VEGF serum concentration in *Deceased* MCT and *Survived* MCT

	N°	VEGF
Deceased	5	pg/mL
mean		103.3
St. Dev.		26.9
median		85.6
Survived	18	
mean		97.6
St. Dev.		27.8
median		85.0
Mann-Whitney test		N.S.

Table 9 Mean, standard deviation and median values of VEGF serum concentration in *Deceased* MCT and *Survived* MCT

	N°	ROMs	BAP	Vitamin E
Deceased	5	U Carr	mmol/L	µmol/L
mean		145.5	2040	25.4
St. Dev.		40.8	386.7	7.3
median		134.8	1949	25.6
Survived	17			
mean		136.5	2007.9	26.5
St. Dev.		31.0	160.2	12.7
median		146.4	1953	23.7
Mann-Whitney test		N.S.	N.S.	N.S.

6.4 DIFFERENCES IN HAEMATOLOGICAL PROFILE BETWEEN GROUPS AND WITHIN MCT SUB GROUPS

Hematology: total white blood cells (WBC) were significantly higher in mesenchimal tumor (*Mesenchimal tumors* group) compared to mast cell tumors (*Mast cell tumors* group) (17.6±15.3 10^9/L and 8.8±3.6 10^9/L, respectively). Platelets count (PLT) was significantly higher in epithelial tumors than in mast cell tumor (Table 10). No statistical significant differences were observed for the other parameters between groups. Within the MCT sub groups total WBC were higher in *Deceased* patients than in *Survived* (Table 12). Red blood cells (RBC) were lower in *Clinical stage 2 & 4* MCT than *Clinical stage 1 & 3* MCT and PLT vice versa (Table 11). No statistical significant differences were observed for the other parameters.

Table 10 Mean and standard deviation of haematological parameters between groups.

	WBC (6-17) 10^9/L	**RBC** (5.5-7.9) 10^9/L	**Hgb** (12-18.5) 10^{12}/L	**Hct** 37-55 %	**MCV** 60-76 fL	**MCH** 20-27 pg	**MCHC** 32-38.5 g/dL	**RDW** 12-16 %	**PLT** 150-400 10^9/L	**MPV** 7.9-12 fL	**Pct** 0.15-0.30 %
Epithelial(n=12)											
Mean	10.0 AB	6.3	14.3	42.2	66.9	22.6	33.8	12.5	352.9A	10.6	0.38°
St. dev.	2.3	1.1	2.8	7.7	3.3	1.5	1.3	1.1	128.8	0.8	0.11
MCT (n=24)											
Mean	8.8 B	6.3	14.6	43.3	68.0	23.0	33.7	12.6	257.7B	11.0	0.28B
St. dev.	3.6	0.9	2.2	6.3	3.5	1.6	1.1	0.7	65.8	0.8	0.07
Mesenchimal (n=10)											
Mean	17.6 A	6.0	14.0	41.2	68.5	23.3	34.0	13.6	279.0AB	11.1	0.31AB
St. dev.	15.3	1.4	3.5	9.9	3.5	1.4	1.9	2.8	93.1	1.1	0.10
One way ANOVA	P=0,009	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	P=0.019	n.s.	P=0.016

Tukey's pairwise comparisons: A≠B (P<0.05)

Table 11 Mean and standard deviation of haematological parameters in *Deceased* MCT and *Survived* MCT

	WBC (6-17) 10^9/L	**RBC** (5.5-7.9) 10^9/L	**Hgb** (12-18.5) 10^{12}/L	**Hct** 37-55 %	**MCV** 60-76 fL	**MCH** 20-27 pg	**MCHC** 32-38.5 g/dL	**RDW** 12-16 %	**PLT** 150-400 10^9/L	**MPV** 7.9-12 fL	**Pct** 0.15-0.30 %
Deceased (n=5)											
Mean	13.3	5.7	13.4	39.7	69.2	23.3	33.6	12.6	207.0	11.5	0.24
St. dev.	5.3	0.8	2.3	6.5	4.7	1.7	1.4	0.7	65.6	1.2	0.07
Survived (n=18)											
Mean	7.6	6.5	15.0	44.4	67.8	23.0	33.8	12.6	271.7	10.9	0.30
St. dev.	1.8	0.9	2.1	6.3	3.2	1.6	1.1	0.7	62.3	0.7	0.06
2 sample t-test	P=0,026	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Tukey's pairwise comparisons: A≠B (P<0.05)

Table 12 Mean and standard deviation of haematological parameters in *Clinical stage 1 & 3* MCT and *Clinical stage 2 & 4* MCT

	WBC (6-17) 10^9/L	**RBC** (5.5-7.9) 10^9/L	**Hgb** (12-18.5) 10^{12}/L	**Hct** 37-55 %	**MCV** 60-76 fL	**MCH** 20-27 pg	**MCHC** 32-38.5 g/dL	**RDW** 12-16 %	**PLT** 150-400 10^9/L	**MPV** 7.9-12 fL	**Pct** 0.15-0.30 %
STAGE 1+3 (n=17)											
Mean	7.70	6.57	15.15	44.74	67.65	22.92	33.82	12.53	275.3	10.93	0.30
St. dev.	1.82	0.94	2.15	6.30	3.28	1.64	1.12	0.64	62.3	0.68	0.06
STAGE 2+4 (n=7)											
Mean	11.50	5.76	13.44	39.74	6.86	23.14	33.54	12.79	215.0	11.41	0.24
St. dev.	5.30	0.70	1.92	5.35	4.02	1.50	1.16	0.87	57.0	1.1	0.06
2 sample t-test	n.s.	P=0,033	n.s. (P=0.081)	n.s. (P=0.070)	n.s.	n.s.	n.s.	n.s.	P=0.041	n.s.	n.s.

Tukey's pairwise comparisons: A≠B (P<0.05)

6.5 DIFFERENCES IN BIOCHEMICAL PROFILE BETWEEN GROUPS AND WITHIN MCT SUB GROUPS

No significant differences were observed within the biochemical profile between groups except for total protein that were significantly higher in MCT and mesenchimal tumor than in mesenchimal ones (Table 13). No statistical differences were observed when *Deceased* MCT group was compared with *Survived* MCT except for prothrombin time (PT) that was significantly lower in deceased patients than in survived (Table 13). When *Clinical stage 1 & 3* MCT and *Clinical stage 2 & 4* MCT were compared, total bilirubin was significantly higher in patients with stage 2&4 than 1&3 and PT was significantly higher in stage 1&3 than in stage 2&4 (Table 15).

Table 13 Mean and standard deviation of biochemical parameters between groups.

	Total Ca Mg/dL (8-11)	Phosphorus Mg/dl (2.9-5)	Total protein g/dl (5.5-7.7)	Albumin g/dL(2.5-4)	ALKP U/L (77-200)	GGT U/L (2.9-12.6)	ALT U/L (31-95)	Urea Mg/dL (20-60)	Creatinin Mg/dL(0.8-1.5)	Total bilirubin Mg/dL0.13-0.3
Epithelial N=12										
Mean	9.03	3.95	5.62 B	2.81	251.3	11.29	70.84	34.17	1.11	0.35
St. dev.	0.79	1.25	0.97	0.45	186.4	7.02	29.13	20.96	0.55	0.33
MCT N=24										
Mean	9.25	4.15	6.81 A	2.92	225.8	12.35	64.15	28.31	0.97	0.38
St. dev.	1.04	1.09	0.76	0.47	387.1	6.83	33.96	7.86	0.15	0.34
Mesenchimal N=10										
Mean	8.92	4.06	6.70 A	2.74	403.0	13.53	92.50	36.07	0.97	0.23
St. dev.	1.02	0.67	0.78	0.58	480.0	6.88	63.80	15.74	0.18	0.08
One way ANOVA	n.s.	n.s.	P=0,001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

	Cholesterol Mg/dL (150-265)	Triglycerides Mg/dL (66-110)	Glucose Mg/dL (80-120)	PT Sec (5.2-7.6)	APTT Sec (9-20)
Epithelial N=12					
Mean	220.3	95.23	91.39	6.77	13.02
St. dev.	62.0	100.76	9.38	0.83	1.98
MCT N=24					
Mean	244.6	79.76	97.20	6.83	13.52
St. dev.	89.7	46.23	13.27	0.88	2.43
Mesenchimal N=10					
Mean	195.4	59.19	89.79	7.52	13.80
St. dev.	108.3	23.98	15.88	0.97	2.91
One way ANOVA	n.s.	n.s.	n.s	n.s.	n.s.

Tukey's pairwise comparisons: A≠B (P<0.05)

Table 14 Mean and standard deviation of biochemical parameters in *Deceased* MCT and *Survived* MCT

	Total Ca Mg/dL (8-11)	Phosphorus Mg/dl (2.9-5)	Total protein g/dl (5.5-7.7)	Albumin g/dL(2.5-4)	ALKP U/L (77-200)	GGT U/L (2.9-12.6)	ALT U/L (31-95)	Urea Mg/dL (20-60)	Creatinin Mg/dL(0.8-1.5)	Total bilirubin Mg/dL0.13-0.3
Deceased N=5										
Mean	8.94	4.24	6.86	3.01	234.22	10.08	68.24	28.38	0.89	0.73
St. dev.	0.53	1.14	0.79	0.60	152.54	4.30	14.87	4.00	0.21	0.67
Survived N=18										
Mean	9.40	4.17	6.82	2.93	215.44	12.92	61.75	28.20	1.00	0.28
St. dev.	1.13	1.12	0.80	0.44	442.64	7.54	38.30	8.93	0.14	0.08
2 sample t-test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Tukey's pairwise comparisons: A≠B (P<0.05)

	Cholesterol Mg/dL (150-265)	Triglycerides Mg/dL (66-110)	Glucose Mg/dL (80-120)	PT Sec (5.2-7.6)	APTT Sec (9-20)
Deceased N=5					
Mean	249.70	85.82	104.68	6.20	13.14
St. dev.	91.08	58.26	16.45	0.48	1.39
Survived N=18					
Mean	238.16	76.39	95.68	7.02	13.58
St. dev.	91.66	44.90	12.24	0.91	2.73
2 sample t-test	n.s.	n.s.	n.s.	P=0.020	n.s.

Tukey's pairwise comparisons: A≠B (P<0.05)

Table 15 Mean and standard deviation of biochemical parameters in *Clinical stage 1 & 3* MCT and *Clinical stage 2 & 4* MCT

	Total Ca Mg/dL (8-11)	Phosphorus Mg/dl (2.9-5)	Total protein g/dl (5.5-7.7)	Albumin g/dL(2.5-4)	ALKP U/L (77-200)	GGT U/L (2.9-12.6)	ALT U/L (31-95)	Urea Mg/dL (20-60)	Creatinin Mg/dL(0.8-1.5)	Total bilirubin Mg/dL0.13-0.3
ST 1+3 N=17										
Mean	9.45	4.15	6.83	2.87	222.7	12.51	61.11	27.79	0.99	0.266
St. dev.	1.14	1.15	0.81	0.39	455.2	7.56	39.38	9.03	0.14	0.071
ST 2+4 N=7										
Mean	8.77	4.15	6.74	3.04	233.3	11.94	71.56	29.57	0.94	0.646
St. dev.	0.54	1.01	0.68	0.65	148.1	5.11	14.02	4.12	0.19	0.568
2 sample t-test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	P=0,031

Tukey's pairwise comparisons: A≠B (P<0.05)

	Cholesterol Mg/dL (150-265)	Triglycerides Mg/dL (66-110)	Glucose Mg/dL (80-120)	PT Sec (5.2-7.6)	APTT Sec (9-20)
ST 1+3 N=17					
Mean	234.5	76.65	97.21	7.02	13.77
St. dev.	93.1	46.27	10.71	0.94	2.69
ST 2+4 N=7					
Mean	269.0	87.30	97.17	6.37	12.90
St. dev.	81.9	48.88	19.22	0.51	1.63
2 sample t-test	n.s.	n.s.	n.s.	P=0.040	n.s.

Tukey's pairwise comparisons: A≠B (P<0.05)

6.6 PROPORTION OF CANCER PATIENTS OUTSIDE THE REFERENCE RANGE (IRON PROFILE, VEGF, OXIDATIVE STRESS PATTERN)

In cancer groups the 50% of the patients affected by mesenchimal tumors showed hyposideremia while this occurred in 16.7% of epithelial tumor and just 8.3% of MCT; hyposideremia occurred also in 10% of the healthy patients. Hypersideremia was rare, happening in 8.3% of MCT and 8.3% of epithelial tumor. Ferritin was low in 50% of the healthy patients as well in 20% of the *Non neoplastic diseases* group and within the cancer groups MCT showed the highest variability with 25% of the patients under the reference range and 12.5% above the reference range. ROMs values of the three oncologic groups were always higher than the normal reference interval derived from (Pasquini et al 2008). BAP was always in the reference range in all the cancer groups as well as in the other groups. The reference range for vitamine E was calculated based on the healthy control group (19.9-35.9 µmol/L) and was found to be under the reference range in 80% of the mesenchimal tumor as well as the 80% of the *Non neoplastic diseases* group. MCTs and epithelial tumors showed a Vitamin E under the reference range in the 39.1 and 33.3% respectively. Within the cancer groups vitamin E was found to be increased in the 17.4 % of the MCTs and 8.3% of the epithelial tumor. VEGF of the cancer groups and Non neoplastic disease was compared with the healthy patients and was found to be increased in 30% of MCT, 20% of the mesenchimal tumor and 16.7% of the epithelial tumour. 60% of the *Non neoplastic disease* group patients had elevation in VEGF serum concentration (Table 16).

Table 16 Proportion of cancer patients below and above the reference range (iron profile, VEGF, Oxidative stress pattern)

	Iron µg/dL		Ferritin ng/mL		UIBC µp/dL		TIBC µp/dL		Saturation %	
Out of range	<95	>226	<85	>286	<182	>306	<318	>479	<28.2	>56.8
MCT	2/24 (8.3%)	2/24 (8.3%)	6/24 (25%)	3/24 (12.5%)	5/24 (20.8%)	2/24 (8.3%)	5/24 (20.8%)	2/24 (8.3%)	2/24 (8.3%)	4/24 (16.7%)
MESEN	5/10 (50.0%)	0/10 (0%)	1/10 (10%)	0/10 (0%)	4/10 (40%)	0/10 (0%)	5/10 (50%)	0/10 (0%)	5/10 (50%)	0/10 (0%)
EPIT	2/12 (16.7%)	1/12 (8.3%)	2/12 (16.7%)	1/12 (8.3%)	3/12 (22.2%)	1/12 (8.3%)	2/12 (16.7%)	1/12 (8.3%)	2/12 (16.7%)	0/12 (0%)
CTR H	1/10 (10%)	0/10 (0%)	5/10 (50%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/10 (10%)	0/10 (0%)	1/10 (10%)	0/10 (0%)
CTR PAT	0/10 (0%)	0/10 (0%)	2/10 (20%)	0/10 (0%)	3/10 (30%)	0/10 (0%)	3/10 (30%)	0/10 (0%)	1/10 (10%)	0/10 (0%)

	ROMs U-Carr		BAP mmol/L		Vit. E µmol/L		VEGF pg/mL	
Out of range	<56.3*	>91.1*	<1305*	>3587*	<19.9§	>35.9§	<46.6§	>117.5§
MCT	0/23 (0%)	23/23 (100%)	0/23 (0%)	0/23 (0%)	9/23 (39.1%)	4/23 (17.4%)	0/23 (0%)	7/23 (30.4%)
MESEN	0/10 (0%)	10/10 (100%)	0/10 (0%)	0/10 (0%)	8/10 (80%)	0/10 (0%)	1/10 (10%)	2/10 (20%)
EPIT	0/12 (0%)	12/12 (100%)	0/12 (0%)	0/12 (0%)	4/12 (33.3%)	1/12 (8.3%)	0/12 (0%)	2/12 (16.7%)
CTR H	1/10 (10%)	0/10 (0%)	0/10 (0%)	2/10 (20%)	0/10 (0%)	0/10 (0%)	0/10 (10%)	0/10 (0%)
CTR PAT	1/10 (10%)	5/10 (50%)	1/10 (10%)	1/10 (10%)	8/10 (80%)	0/10 (0%)	0/10 (0%)	6/10 (60%)

*Reference interval was obtained from Pasquini et al 2008 (mean±1.96standard deviation)

§Lower and higher values of the *Healthy* control group

Within the MCT *Clinical stage 1 & 3* MCT and *Clinical stage 2 & 4* MCT groups, hypersideremia and hyposideremia occurred just in *Clinical stage 2 & 4* MCT group. Ferritin was low in the 27.8% of the *Clinical stage 1 & 3* MCT. As reported before 100% of the of the MCT showed increase of ROMs and normal BAP. Vitamin E was reduced in 41% of the *Clinical stage 1 & 3* compared with the 28.6% of the *Clinical stage 2 & 4.*

VEGF was found to be higher in 42.8% of the stage *Clinical stage 2 & 4* compared with the 22.2% of the *Clinical stage 1 & 3.*

Table 17 Proportion of *Clinical stage 1 & 3* MCT and *Clinical stage 2 & 4* MCT below and above the reference range (iron profile, VEGF, Oxidative stress pattern)

	Iron µg/dL		Ferritin ng/mL		UIBC µp/dL		TIBC µp/dL		Saturation %	
Out of range	<95	>226	<85	>286	<182	>306	<318	>479	<28.2	>56.8
Stage 1+3	0/18 (0%)	0/18 (0%)	5/18 (27.8%)	2/18 (11.1%)	4/18 (22.2%)	2/18 (11.1%)	4/18 (22.2%)	2/24 (8.3%)	1/18 (5.5%)	3/18 (16.7%)
Stage 2+4	2/7 (28.6%)	2/7 (28.6%)	1/7 (14.3%)	1/7 (14.3%)	1/7 (14.3%)	0/7 (0%)	2/7 (28.6%)	2/7 (28.6%)	1/7 (14.3%)	1/7 (14.3%)

	ROMs U-Carr		BAP mmol/L		Vit. E µmol/L		VEGF pg/mL	
Out of range	<56.3*	>91.1*	<1305*	>3587*	<19.9§	>35.9§	<46.6§	>117.5§
Stage 1+3	0/17 (0%)	17/17 (100%)	0/17 (0%)	0/17 (0%)	7/17 (41.2%)	3/17 (17.6%)	0/18 (0%)	4/18 (22.2%)
Stage 2+4	0/7 (0%)	7/7 (100%)	0/7 (0%)	0/7 (0%)	2/7 (28.6%)	1/7 (14.3%)	0/7 (10%)	3/7 (42.8%)

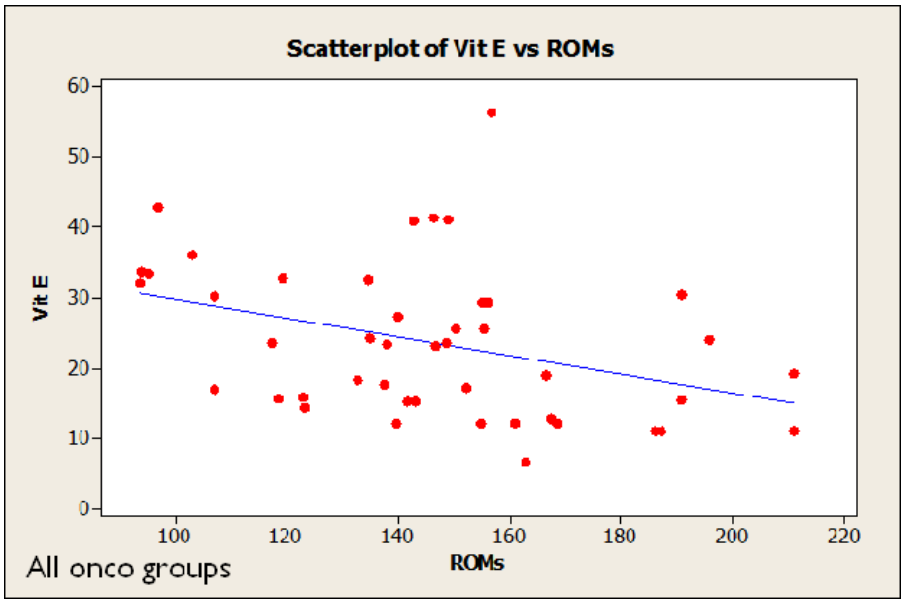
*Reference interval was obtained from Pasquini et al 2008 (mean±1.96standard deviation)

§Lower and higher values of the CTR H group

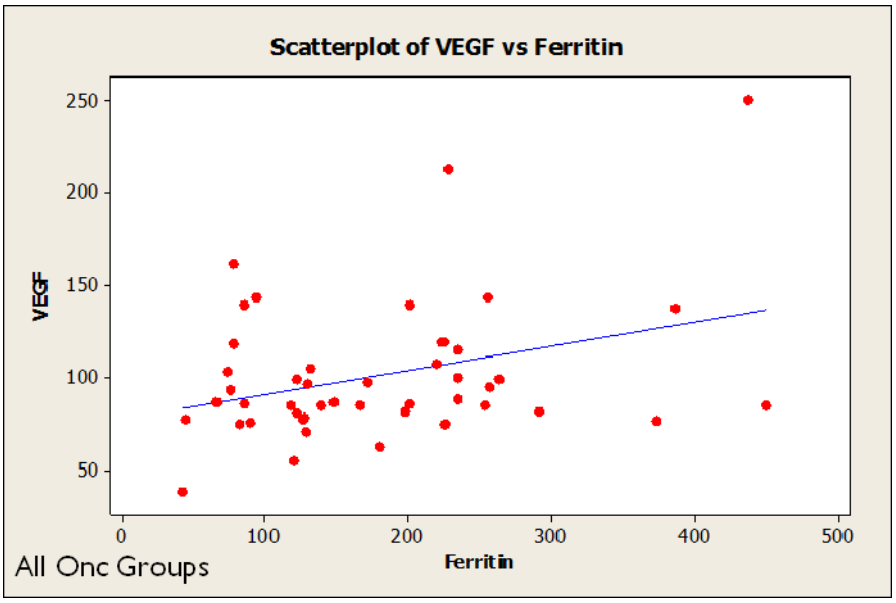
Mortality within 1 year from diagnosis of MCT was higher among dogs belonging to Stages 2+4 compared to those belonging to Stages 1+3: 5/7 (71.4%) and 0/17 (0%), respectively (Fisher's exact test: P<0.001).

6.7 RELATIONS BETWEEN VARIABLES

Scatter plot graphs and Pearson's correlations of the variables between all the oncologic groups were performed and the statistically significant are reported below.

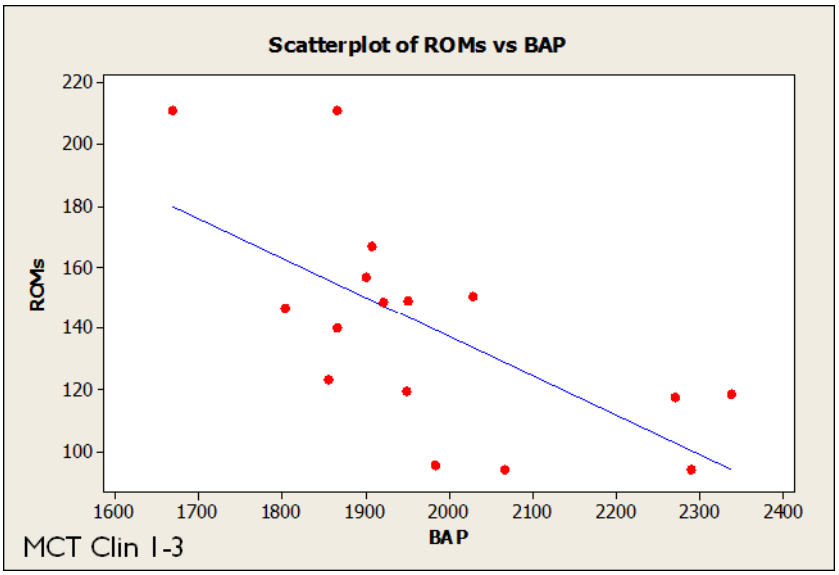


r -0,371; p <0.012

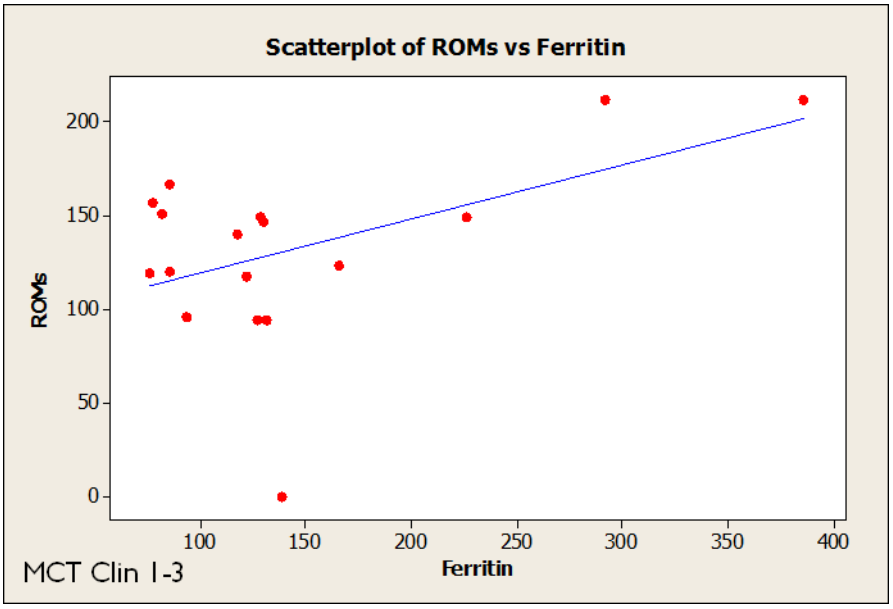


r 0,344; p <0.019

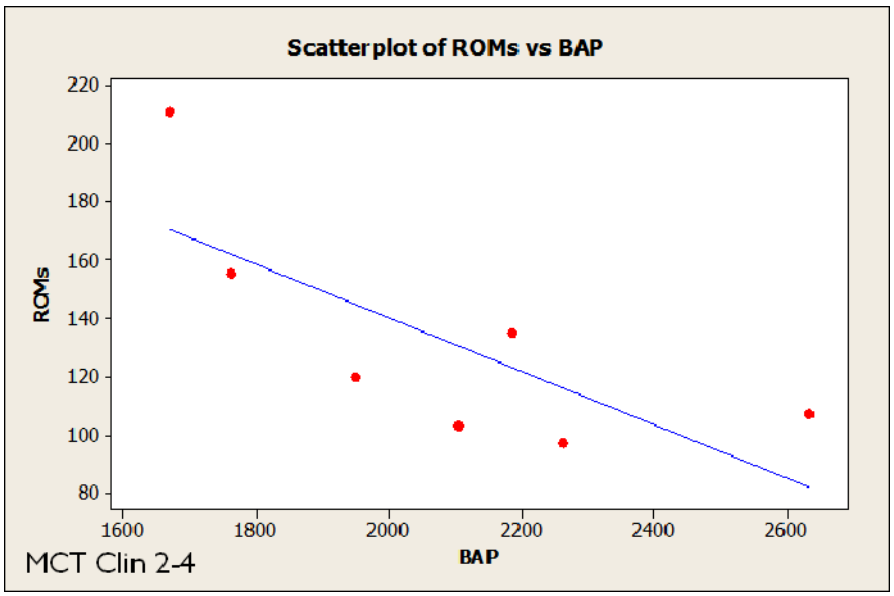
Scatter plot graphs and Pearson's correlations of the variables within MCT sub groups were performed and the statistically significant are reported below.



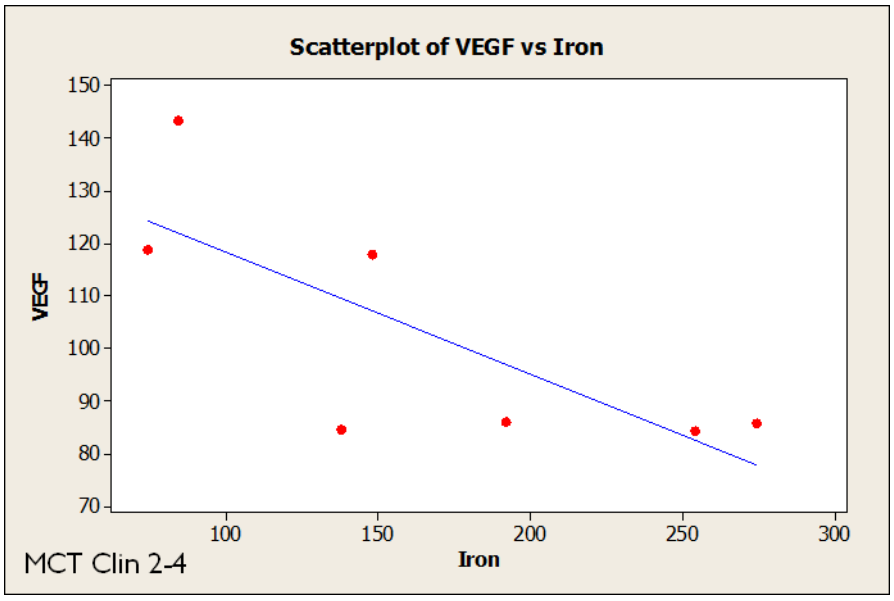
r -0,656; p <0.006



r 0.494; p <0.044



r -0.749; p <0.05



r -0.763; p <0.046

7. DISCUSSION

Aim of this prospective study was to evaluate Iron status, VEGF serum concentration, and oxidative stress pattern thorough ROMs, BAP and Vitamin E serum concentration in canine cancer patients focusing on canine mast cell tumours.

ROMs, BAP and Vitamine E have been evaluated *in vivo* in groups of non Hodgkin's lymphoma canine patients (Vajdovich, 2005; Winter, 2009); in these studies a significant increase of ROMs and reduction of BAP was observed. In our study we enrolled epithelial, mesenchimal and round cell tumours (MCT) observing and increase of ROMs in all the cancer patients when compared with the reference range and our population of healthy dogs. Although none of the oncologic patients showed BAPs values below the reference range (Pasquini, 2008), the values of the healthy dogs were significantly higher than those affected by MCT, epithelial tumours and non neoplastic disease. Patients with mesenchimal tumours despite a significant increase of ROMs had no significant differences in BAP when compared with healthy dogs. This could be related with the heterogeneous and small population of tumour enrolled, and significance maybe reach increasing the number of patients. It is thus possible that the range for healthy animals derived from the study of Pasquini (2008) was particularly wide, despite the fact that it was obtained from dogs belonging to one single breed (Labrador retriever). Although it is expected that an increase of ROMs would be related with a decrease of BAP, this was observed only in MCT tumours group. This result could be due to the small number of epithelial and mesenchimal tumours; moreover have to be

considered that MCTs group was composed by an homogeneous population of cancer, different from the other two groups (epithelial and mesenchimal tumors) where different biological entity were considered together. In our study, Vitamin E was not significantly decreased in cancer patients when compared to the healthy group, although mesenchimal tumours approached significance. Is interesting how Vitamin E was negatively correlated with ROMs when all the cancer patients were considered together; this maybe explained with the fact that not all the substances taking part in the BAP (i.e. Vitamin C, GSH, γ-Tocopherol, ORAC, metallothionein), are affected in the same manner in cancer patients. This was already observed by Winter et al. in canine lymphoma patients were a decrease of Vitamine E was followed by an independent increase of other BAP's components, resulting in a partial compensation of the BAP. However, it is important to note that BAP seems to be still high in cancer patients both in our study than in the ones reported below (Vajdovich, 2005; Winter, 2009) and antioxidant administration might not prove to be clinically beneficial as supplementation of vitamin E or other antioxidants could diminish the efficacy of certain chemotherapeutic agents by reducing the production of ROS. Further studies will be needed to evaluate the relative benefits and risks of antioxidant supplementation.

In vitro studies purposed the role of Vitamin E in prevents mediator release by mast cells (Gueck, 2002; Kempna, 2004) but in our population we observed no significant correlation of Vitamin E serum concentration values and MCT clinical sub stage. Otherwise further studies should be performed including more MCT in high clinical stage (stage 4).

In veterinary medicine there are no studies which used the methods used in this thesis for the evaluation of BAPs and ROMs in cancer patients, thus the ones described early focused on single molecules. In humans, evaluating BAPs and ROMs in a way similar to the one we used in this thesis, it was concluded that oxidative status might be a useful clinical marker for the evaluation of malignant grades and clinical stages of non hodgkin lymphoma (Nojima, 2011). Moreover in another study the level of ROMs were higher in lung cancer patients than in normal subjects, thus ROMs seems to be altered in most of the neoplastic disease (Katsabeki-Katsafli, 2007). The same authors found a significance difference in serum VEGF levels between lung cancer patients and healthy control subjects. Also in our study healthy control patients had a significant lower VEGF serum concentration compared with epithelial and MCT patients. Is interesting how our healthy control group had a detectable VEGF serum level in disagreement with the results of Troy et al (2006) were healthy patients had an undetectable serum VEGF in more than 90% of the cases. This could be related with the different antibodies used for the ELISA assay: canine in this study and human in Troy et al. The present study failed in finding a significant correlation between VEGF and ROMs, although VEGF has been found to be up-regulated by conditions associated with the generation of free radicals and reactive oxygen intermediates (oxidants) (Maulik, 2002). In the study of Wergin et al (2004) serum VEGF in canine patients was found to be related with the malignancy of the neoplastic disease in epithelial and mesenchimal tumours, on the contrary in the study of Patruno et al. (2009) this was not observed in canine MCT were the serum VEGF was not related with histophatological grading and or clinical staging while a relation was present for cytosol VEGF. Our results on serum VEGF in MCT

appeared to be in agreement with Patruno et al. (2009). Concerning iron status it was observed that ferritin was higher in cancer patients as it was in non neoplastic disease compared to the healthy patients. This maybe suggests that ferritin is not a specific marker for cancer and may behave as a positive acute phase protein also in these patients. However ferritin was also positively correlated with VEGF in our cancer patients and this could be related with a role of VEGF not only as a neoangiogenetic factor but maybe also as an acute phase protein. In conclusion, these data add interesting information on iron serum status, serum VEGF and oxidative stress pattern in oncologic patients. Oxidative stress and VEGF appeared both significantly increased in patients with mast cell and epithelial tumors.

RESEARCH ACTIVITIES

ORAL SHORT COMMUNICATION AT INTERNATIONAL SCIENTIFIC MEETINGS

Marconato L, Romanelli G, Stefanello D, Giacoboni C, Bonfanti U, Bettini G, **Finotello R**, Verganti S, Valenti P, Ciaramella L, Zini E. Long term follow-up and predictors of survival in dogs with mammary inflammatory carcinoma: a retrospective analysis of 43 cases. Annual Congress of the European Society of Veterinary Oncology (ESVONC), Budapest 27th - 29th March 2009

Marconato L,Crispino G, **Finotello R**, Mazzotti S, Zini E. La concentrazione sierica di latticodeidrogenasi predice recidiva in cani con linfoma (Serum concentration of lactate dehydrogenase predict relapse in dogs with lymphoma). 62° International congress multisala SCIVAC. Palacongressi della Riviera di Rimini, Rimini, Italy 29-31 May 2009

Finotello R, Sbrana S, Mazzei M, Citi S, Filocamo M, Barsotti G, Cantile C. Un caso di mucopolissaccaridosi tipo VI (Sindrome di Maroteaux-Lamy) in un gatto (A case of mucopolysaccharidosis VI, Maroteaux-Lamy syndrome, in a cat). 62° International congress multisala SCIVAC. Palacongressi della Riviera di Rimini, Rimini, Italy 29-31 May 2009

Finotello R, Lippi I, Meucci V, Marchetti V, Marconato L, Rota A, Cancedda S, Leone VF, Laganga P, Giudi G. Role of glomerular filtration rate (GFR) to predict haematological toxicity in dogs receiving chemotherapy: a preliminary study. Annual Congress of the European Society of Veterinary Oncology (ESVONC), Glasgow 24th – 26th March 2011

POSTER AT INTERNATIONAL SCIENTIFIC MEETINGS

Ressel L, **Finotello R**, Vannozzi I, Innocenti VM, Poli A. Expression of Leptin (OB) and Leptin Receptor (ObR) in normal, hyperplastic and neoplastic canine mammary tissues, European Society of Veterinary Pathology meeting, pp 195-195, Dubrovnik, Croatia, 2008

Finotello R. Marchetti V, Baroni G, Dini F, Citi S, Poli A, Di Lollo S. A case of Lipoblastoma in a dog. 65th International congress multisala SCIVAC. Palacongressi della Riviera di Rimini, Rimini, Italy 28-30 May 2010.

ORAL COMMUNICATION AT NATIONAL SCIENTIFIC MEETINGS (INVITED SPEAKER)

Finotello R. Principali classi di chemioterapici e meccanismi d'azione: sali del platino, antimetaboliti, alcaloidi della vinca (Main classes of chemotherapeutic drugs and mechanism of action: platinum compounds, antimetabolites and vinka alkaloids). Chemioterapia (Chemotherapy), SIONCOV meeting, Cremona, Italy, September 26-27th 2009

Briganti A., **Finotello R**. Effetto stressogeno della chemioterapia in pazienti con port vascolari versus senza (stressful effect of chemotherapy in patient with vascular access port VS without). S.I.I.G.I.A.V 2nd national congress, Pisa, Italy, 26th – 27th November 2010

ORAL SHORT COMMUNICATION AT NATIONAL SCIENTIFIC MEETINGS

Verganti S, Bettini G, Crispino GP, Ciaramella L, **Finotello R**, Turba M.E., Marconato L. Nuovi aspetti della terapia a bersaglio molecolare: mastocitoma CD117 negativo responsivo ad Imatinib. SIONCOV meeting: Cremona, Italy, October 11-12th 2008.

Finotello R, Marchetti V, Nesi G, Arvigo M, Baroni G, Vannozzi I. Ipoglicemia in un cane con tumore delle isole pancreatiche secernente Insulin-Like Growth Factor Type-II (Hypoglycaemia in a dog with a pancreatic islet cell tumour with secretions of the Insulin-like Growth Factor Type-II). SICIV-SIONCOV meeting: Cremona, Italy, 31/01/2009 - 01/02/2009.

Crispino GP, Rossi F, Mazzotti S, **Finotello R**, Ciaramella L, Marconato L. Un caso di gastronoma in un cane (A case of gastronoma in a dog). SIONCOV-SCVI meeting: Cremona, Italy, 31/01/2009 - 01/02/2009.

Finotello R, Verin R, Ressel L, Piccinini R, Poli A. Studio morfologico ed immunoistochimico di un carcinoma mammario in una tigre siberiana, Panthera tigris altaica (Morfological and immunohistochemical study of a mammary carcinoma in siberian tiger, Panthera tigris altaica). SIVASZoo meeting: Udine, Italy 19-20 June 2009

Marchetti V, Bocelli G, Marconato L, **Finotello R**, Guidi G, Podestà A, Cardini G. Pattern delle isoforme della fosfatasi alcalina nei cani oncologici (Alkaline phosphatase isoforms in neoplastic dogs). LXIII SISVET congress: Udine, Italy 16-18th September 2009

Citi S, Mannucci T, **Finotello R**, Sbrana S, Marchetti V. Neoplasie Mixoidi appendicolari nel cane: 5 casi clinici (appendicular myxoid tumors: 5 clinical cases). 17° Annual SICV Congress: Olbia, Italy 24-26th June 2010

PUBBLICATIONS

Marconato L., Comazzi S., Esposito R., Ciaramella L., Crispino G., **Finotello R.** Sarcoma istiocitico disseminato con cellule neoplastiche circolanti in un cane (diffuse histiocytic sarcoma with circulating neoplastic cells in a dog). *Veterinaria* 22 (6), 2008

Crispino G., Rossi F., Mazzotti S., **Finotello R**., Abramo F., Magni G., Marconato L. Un caso di gastronoma in un cane (a case of gastronoma in a dog). *Veterinaria* 23(3), 2009

Marconato L, Romanelli G, Stefanello D, Giacoboni C, Bonfanti U, Bettini G, **Finotello R**, Verganti S, Valenti P, Ciaramella L, Zini E. Predictors of survival in

dogs with mammary inflammatory carcinoma: a retrospective analysis of 43 cases (2003-2008). *JAVMA*, 235(8): 967-72, 2009

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Marconato L, Crispino G, **Finotello R**, Mazzotti S, Zini E. Clinical relevance of seral determination of lactate dehydrogenase levels to predict recurrence in dogs with lymphoma. *JAVMA*, 236 (9): 969-974, 2010

Marconato L, Crispino G, **Finotello R**, Mazzotti S, Zini E. Evaluation of lactate dehydrogenase activity to predict recurrence of lymphoma in dogs. *Vet Rec* 166: 695, 2010

Finotello R, Ressel L, Verin R, Baroni G, Piccinini R, Di Lollo S. Morfological and immunohistochemical study of a mammary carcinoma in siberian tiger, Panthera tigris altaica. *J Zoo Wildl Med*, 42 (1): 134-138, 2011

Finotello R, Marchetti V, Baroni G, Carlucci F, Dini F, Citi S, Arcangeli A, Poli A, Di Lollo S. Lipoblastoma in a dog: clinical, histochemical and immunohistochemical features. *BJVP* 4 (1): 62- 66, 2011

Marchetti V, Giorgi M, Fioravanti A, **Finotello R**, Citi S, Canu B, Orlandi P, Di Desidero T, Danesi R, Bocci G. First-line metronomic chemotherapy in a metastatic model of spontaneous canine tumours: a pilot study. In *press Investigational New Drugs.*

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