UNIVERSITY OF NOVA GORICA
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DIAGNOSTIC AND PROGNOSTIC MARKERS IN CANINE INFLAMMATORY AND NEOPLASTIC HEAD AND NECK CONDITIONS

DISSERTATION

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UNIVERZA V NOVI GORICI
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DIAGNOSTIČNI IN PROGNOSTIČNI MARKERJI PRI PSIH Z VNETNIMI IN NEOPLASTIČNIMI SPREMEMBAMI V PODROČJU GLAVE IN VRATU

DISERTACIJA

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"In the middle of difficulty lies opportunity."

Albert Einstein

ABBREVIATIONS

Akt - Protein kinase B

APC - antigen presenting cells

BOP- bleeding on probing

CBC - complete blood count

CCR7- C-C chemokine receptor type 7

CD21 - cluster of differentiation 21

CD25 - cluster of differentiation 25

CD28 - cluster of differentiation 28

CD39 - cluster of differentiation 39

CD4 - cluster of differentiation 4

$CD4^+$ - cluster of differentiation 4^+ (helper T cells)

CD40 - cluster of differentiation 40

CD44 - cluster of differentiation 44

CD45RA/CD45RO - cluster of differentiation 45RA/cluster of differentiation RO

CD62L - cluster of differentiation 62 Ligand

CD8 - cluster of differentiation 8

$CD8^+$ - cluster of differentiation 8^+ (cytotoxic T cells)

CD80/86 - cluster of differentiation 80/86

CR - complete response

CT - computed tomography

CTV - clinical target volume

CTLA-4 - co-inhibitory receptor cytotoxic T lymphocyte antigen 4

CXCR4 - C-X-C - chemokine receptor type 4

DNA - deoxyribonucleic acid

EDTA - ethylene-diamine-tetra-acetic-acid

EGFRs - epidermal growth factor receptors

EOS – eosinophils

FACS - fluorescence- activated cell sorter

FAK - focal adhesion kinase

FBS - fetal bovine serum

FC- flow cytometry

FGF - fibroblast growth factor

FGFR3 - fibroblast growth factor receptor 3

FJK-16s - anti- mouse/rat Foxp3 (forkhead box P3)

FKH - forkhead

Flt-3 - Fms-like tyrosine 3

FOXP3 - forkhead family transcription factor 3

GI - gingival index

GITR - glucocorticoid-induced tumor necrosis factor receptor (TNFR) family related gene

HIF-1- hypoxia-inducible factor 1

HIF-1α - hypoxia inducible factor 1, α subunit

HLA-DR - human leucocyte antigen

HN - head and neck

HNC - head and necl conditions

HNSCC - head and neck squamous cell carcinoma

HRE - hypoxia response element

IL-10 - interleukin 10

IL-12 - interleukin 12

IL-1β - interleukin 1 beta

IL-2 - interleukin 2

IL-4 - interleukin 4

IHC - immunohistochemical score

INFγ - interferon gamma

IPEX - Immune Dysregulation Polyendocrinopathy Enteropathy X-linked Syndrome

iTR 35 - IL-35 producing $CD4^+$ T cells

KDa - kilo Dalton

Kit - tyrosine kinase protein Kit

LAG3 - lymphocyte-activation gene 3

LYM - lymphocytes

Lyn - tyrosine kinase Lyn

LZ - leucine zipper

MAPK - mitogen-activated protein kinases

MCH - mean corpuscular haemoglobin

MCHC - mean corpuscular haemoglobin concentration

MCV - mean corpuscular volume

MHC II - major histocompatibility complex II

MPV - mean platelet volume

MPV/PLT - mean platelet volume to platelet ratio

MM - malignant melanoma

mRNA - messenger ribonucleic acid

NEU - neutrophils

NHNC - neoplastic head and neck conditions

N/L ratio - neutrophil to lymphocyte ratio

NFAT - nuclear factor of activated T cells

NFκB - nuclear factor kappa B

NK cells - natural killer cells

NO - nitric oxide

NSAIDs - non-steroidal anti-inflammatory drugs

PBMC - peripheral blood mononuclear cells

PBS - phosphate buffered saline

PBS/BSA - phosphate buffered saline/bovine serum albumin

PCNA - proliferating cell nuclear antigen

PCK - ploščato celični karcinom

P/L ratio - platelet to lymphocyte ratio

PD - progressive disease

PD - periodontal disease

PD0 - periodontal disease 0

PD 1 - periodontal disease 1

PD 2 - periodontal disease 2

PD 3 - periodontal disease 3

PD 4 - periodontal disease 4

PD-1 - programmed cell death protein 1

PDGF - platelet-derived growth factor

PDGFRs - platelet-derived growth factor receptors

PDW - platelet distribution width

PI - periodontal index

PIK3 - phosphatidylinositol 3-kinase

PLC - phospholipase C

PLCR - platelet large cell ratio

PLCRi - adjusted platelet large cell ratio index

PLT – platelets

PPD - periodontal probing depth

PR- partial response

RBC - red blood cells

RDW-CV - red cell distribution width expressed as a coefficient of variation

RDW-SD - red cell distribution width standard deviation

ROS - reactive oxygen species

RTKs - receptor tyrosine kinases

SAR - sarcoma

SCC(s) - squamous cell carcinoma(s)

SNSCC - sino-nasal squamous cell carcinoma

Stage 1 PD - stage 1 periodontal disease

Stage 2 PD - stage 2 periodontal disease

Stage 3 PD - stage 3 periodontal disease

Stage 4 PD - stage 4 periodontal disease

SD - stable disease

SD - standard deviation

TGF-β - tumour growth factor beta

Th cells - helper T cells

Th17 - helper T cells 17

Th2 - helper T cells 2

Th3 - T helper 3 cells

TNF-α - tumour necrosis factor alfa

Tr1 - T regulatory type 1 cells

Tregs - regulatory T cells

TTP - time to progression

TSP1- trombospodonin 1

VEGF - vascular endothelial growth factor

VEGFR-1 (Flt-1) - vascular endothelial growth factor receptor 1 (Fms-Related Tyrosine

VEGFR-2 - vascular endothelial growth factor receptor 2

VEGFR-2 (FLK-1/KDR) - vascular endothelial growth factor receptor 2 (Fetal Liver Kinase 1/ Kinase Insert Domain

VEGFR-3 (Flt-4) - vascular endothelial growth factor receptor 3 (Fms-Related Tyrosine

VRTOG - veterinary radiation treatment oncology group

ZF - zinc finger

WBC - white blood cells

FIGURES

TABLES

TABLE OF CONTENTS

ABSTRACT

Several different types of conditions with inflammatory and neoplastic background affect the anatomical localities of the head and neck in a dog. It is a well-recognized fact that these conditions affect the quality of life, as they are associated with various degree of regional dysfunction, have systemic effects and can be direct or indirect cause of death. Unfortunately, many dogs with both inflammatory and neoplastic head and neck conditions are presented at an advanced stage of the disease which can have significant impact on treatment strategies. The identification of biomarkers is advisable to enhance effective staging, grading and prognostication, which will in turn more accurately direct recommendations for therapy.

These biomarkers can theoretically help to distinguish between inflammatory and neoplastic conditions, justify the management of patients more accurately and potentially predict the prognosis and the survival of patients.

It was our interest to investigate the diagnostic and prognostic value of complete blood count parameters and indices in dogs with head and neck conditions of inflammatory and neoplastic origin, regulatory T cells in dogs with periodontal disease, immunohistochemical (Ki-67 and VEGFR-2) and histopathological biomarkers in dogs with inflammatory and neoplastic head and neck conditions and to evaluate the effectiveness of an accelerated radiotherapy protocol for the treatment of advanced canine HNSCC.

We have proved that the parameters investigated might serve as important supportive diagnostic and/or prognostic biomarkers which might help to improve the treatment strategies of both inflammatory and neoplastic head and neck conditions in dogs and that the accelerated chemoradiotherapy protocol represents an effective alternative treatment option for dogs with advanced HNSCC.

Key words: biomarkers, dogs, periodontal disease, regulatory T cells, head and neck tumours, complete blood count, radiotherapy, vascular endothelial growth factor receptor 2

IZVLEČEK

DIAGNOSTIČNI IN PROGNOSTIČNI MARKERJI PRI PSIH Z VNETNIMI IN NEOPLASTIČNIMI SPREMEMBAMI V PODROČJU GLAVE IN VRATU

Pri psih se v področju glave in vratu pojavljajo številne vnetne in tumorske bolezni. Znano je, da le-te vplivajo na kakovost življenja, saj so povezane z različnimi stopnjami tako področnih kot sistemskih sprememb in so lahko neposredni ali posredni vzrok smrti. Na žalost so mnogi psi tako z vnetnimi kot neoplastičnimi procesi v področju glave in vratu zdravljeni v napredovalih stadijih bolezni, in prav to dejstvo lahko pomembno vpliva tako na izbiro strategije kot izida zdravljenja.

Določanje biomarkerjev je priporočljivo, saj z njihovo pomočjo lahko učinkoviteje določamo stopnjo bolezni, vrednotimo potek bolezni in napovedujemo prognozo. Biomarkerji lahko pomagajo razločevati med vnetnimi in tumorskimi boleznimi.

Doktorska disertacija je razdeljena v štiri sklope in vključuje proučevanje diagnostične in prognostične vrednosti parametrov in indeksov krvne slike pri psih z vnetnimi in tumorskimi obolenji glave in vratu, regulatornih T celic pri psih z različnimi stopnjami parodontalne bolezni ter imunohistokemijskih (Ki-67 in VEGFR-2) in patohistoloških markerjev pri psih z vnetnimi in tumorskimi obolenji glave in vratu. Prav tako smo spremljali učinkovitost pospešenega kemoradioterapevtskega protokola za zdravljenje napredovalih oblik ploščatoceličnega karcinoma (PCK) glave in vratu pri psih.

Dokazali smo, da imajo proučevani biomarkerji pomembno diagnostično in prognostično vrednost ter lahko znatno prispevajo k izboljšanju metod zdravljenja, tako vnetnih kot neoplastičnih procesov v področju glave in vratu pri psih, kakor tudi učinkovitost pospešenega kemoradioterapevtskega protokola za zdravljenje napredovalih oblik PCK v področju glave in vratu pri psih.

Ključne besede: biomarkerji, psi, parodontalna bolezen, regulatorne T celice, tumorji glave in vratu, krvna slika, radioterapija, receptor 2 za vaskularni endotelni rastni faktor

1 RATIONALE OF THE STUDY

The rationale of the doctoral thesis was to investigate clinical, haematological, cytometric, histopathological and immunohistochemical markers in dogs with inflammatory and neoplastic head and neck conditions as well as to present the treatment outcome of dogs with advanced canine HNSCC treated with an accelerated chemoradiotherapy protocol.

The CBC with differential is the commonest laboratory test performed in both human and veterinary medicine in the diagnosis of infectious, inflammatory, neoplastic and immune-mediated diseases. The CBC and its calculated indices (neutrophil to lymphocyte ratio (N/L), platelet to lymphocyte ratio (P/L), mean platelet volume to platelet ratio (MPV/PLT) and adjusted platelet large cell ratio index (PLCRi)) are considered reliable, relatively inexpensive, reproducible and standardized but often underestimated and not utilized. In the first part of the thesis, we focused on the identification of the differences between CBC parameters and CBC indices between canine inflammatory and neoplastic head and neck conditions and assessment of CBC indices as potential distinguishing biomarkers between head and neck conditions of inflammatory and neoplastic origin. Its potential prognostic value in dogs with head and neck neoplasia was also assessed.

The second aim of the thesis was to measure the systemic and locoregional lymph node levels of Tregs along with other lymphocyte subpopulations ($CD4^+$, $CD8^+$ and $CD5^+$ T cell subsets and $CD21^+$ B cells) in dogs affected with periodontal disease (PD), more specifically, stage 1 PD (gingivitis) and stage 3,4 PD (moderate to advanced periodontitis) detected by flow cytometry (FC). T cells play a central role in cellular immunity in a wide range of pathologic conditions, including inflammatory, neoplastic, infectious and (auto)-immune diseases with alterations in their frequency and function detected both locally and systemically. The data

regarding systemic levels of Tregs in human patients with PD is limited and no data are published regarding the systemic role of these cells in dogs with PD. Our aim was to investigate if the differences of Tregs in the peripheral blood and regional lymph nodes along with other lymphocyte subpopulations ($CD4^+$, $CD8^+$ and $CD5^+$ T cell subsets and $CD21^+$ B cells) between dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis) exist indicating their involvement in the pathogenesis of PD. Targeting Tregs, with, for instance, specific cytokine profile, might help to prevent the development of PD or improve the treatment strategies and/or treatment outcome of PD in both humans and in dogs.

In the third part of the thesis the clinical (results of clinical examination, blood works, computed tomography), histopathological (grading, presence of necrosis, mitotic index) and immunohistochemical parameters, Ki-67 and vascular endothelial growth factor receptor 2 (VEGFR-2) were determined, evaluated and compared between canine inflammatory and neoplastic benign and malignant head and neck conditions. A correlation of parameters investigated was compared with the overall survival of canine patients with neoplastic head and neck conditions. Better understanding of the biology will help to elucidate new aspects of canine head and neck neoplasia biological and clinical behaviour, which is essential as it might help to guide the therapy and develop new, more effective treatment strategies.

Patients with advanced HN tumours, in both human and veterinary medicine, represent a therapeutic challenge as their prognosis, mainly due to ineffectiveness to traditional therapies, is generally considered to be poor. Local recurrence of advanced canine HNSCC is common despite aggressive treatment. Our fourth aim was to investigate the efficacy of accelerated chemoradiotherapy protocol for these types of HN neoplasia, planned, executed with linear accelerator and followed-up with computed tomography.

It is a well-known fact that canine diseases, like periodontal disease and spontaneous tumours, present a good model to study development, progression and assessment of therapeutic modalities for human diseases and this translational aspect is the final goal of the presenting study. We expect our findings will contribute to a better understanding of the possible underlying mechanisms of both, periodontal diseases and neoplastic HN conditions in dogs.

2 OBJECTIVES OF THE DOCTORAL DISSERTATION

1. Investigate and compare the CBC parameters and CBC calculated indices (N/L, P/L, MPV/PLT in PLCRi) between healthy dogs, dogs with stage 3,4 PD (moderate to advanced periodontitis) and canine patients with neoplastic HN conditions and to correlate the CBC indices of canine patients with neoplastic HN conditions with the overall survival.

2. Investigate and compare the percentages of Tregs ($CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$) and other lymphocyte subpopulations ($CD4^+$, $CD8^+$ and $CD5^+$ T cell subsets and $CD21^+$ B cells) in the peripheral blood and Tregs ($CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$) in regional lymph nodes in dogs with stage 1 PD (gingivitis) and in dogs with stage 3,4 PD (moderate to advanced periodontitis) using flow cytometric analysis.

3. Investigate and compare the clinical results (clinical examination, blood works, computed tomography), histopathological (grading, presence of necrosis, mitotic index) and immunohistochemical biomarkers, Ki-67 and VEGFR-2, in dogs with inflammatory and neoplastic benign and malignant HN conditions and correlate the measured biomarkers with the overall survival.

4. Assess the treatment efficacy of an accelerated chemoradiotherapy protocol for advanced canine HNSCC.

3 HYPOTHESES

1. We hypothesize that there are significant differences in CBC parameters and CBC indices (N/L, P/L, MPV/PLT in PLCRi) between healthy dogs, dogs with stage 3,4 PD (moderate to advanced periodontitis) and canine patients with neoplastic HN conditions. CBC indices can serve as potential distinguishing biomarkers between canine HN conditions of inflammatory and neoplastic origin.

2. We hypothesize that there are significant differences in the percentages of Tregs ($CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$) and other lymphocyte subpopulations ($CD4^+$, $CD8^+$ and $CD5^+$ T cell subsets and $CD21^+$ B cells) between dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis) with respect to systemic circulation, revealing their potential involvement in the pathogenesis of PD.

3. We hypothesize that the expression of Ki-67 and VEGFR-2 is higher in advanced neoplastic HN conditions. Increased expression of Ki-67 and VEGFR-2 correlates with worse treatment outcome and overall survival.

4. We hypothesize that an accelerated chemoradiotherapy protocol is an effective treatment modality for advanced canine HNSCC.

4 INTRODUCTION

4.1 Complete blood count parameters and indices in canine inflammatory and neoplastic head and neck conditions

4.1.1 Role of inflammation in progression of tumours

Several studies demonstrated the important role of inflammation in progression of different types of tumours (Balkwill & Mantovani, 2001; Galon et al., 2007; Mantovani et al., 2008). The tumour microenvironment is a complex composition of tumour cells surrounded by stroma (containing mesenchymal and epithelial cells, fibroblasts, pericytes), innate (neutrophils, macrophages, dendritic cells or mast cells) and adaptive immune cells (lymphocytes T and B) (Coussens & Werb, 2002; Grivennikov, 2010). The neoplastic inflammatory component, therefore, includes a diverse population of cells that each, with their individual function, participate in neoplastic processes by promoting tumour cell proliferation, survival and migration (Balkwill & Mantovani, 2001; Coussens & Werb, 2002). The cells exhibit their activity by direct cell-to-cell contacts or by the production of pro-inflammatory and anti-inflammatory cytokines/chemokines, which attract leukocytes (Coussens & Werb, 2002). Pro-inflammatory cytokines provoke inflammation and angiogenesis, which are in turn stimulating the growth of tumours. On the opposite, low level of cytokines/chemokines is usually associated with constrained growth of tumours (Coussens & Werb, 2002; Fridlender & Albeida, 2012). Increased infiltration of neutrophils, for instance, in response to an altered balance of pro-inflammatory and anti-inflammatory cytokines, is associated with cytotoxicity, angiogenesis and tumour progression (Coussens & Werb, 2002). The interaction between tumour cells and platelets in neoplastic conditions exists as well as platelets have been demonstrated to be able to stimulate haematogenous spread of tumour cells, tumour

cell adhesion, invasion and angiogenesis (Coussens & Werb, 2002; Dubernard et al., 1997; Kaplan et al., 1979; Quian & Thuszyanski, 1996).

4.1.2 Complete blood count parameters and indices in inflammatory and neoplastic processes

CBC indices like neutrophil or platelet to lymphocyte ratios (Bhatti et al., 2010; Kwon et al., 2012) have been, with other CBC parameters and traditional prognostic factors (e.g. tumour size, histologic grade, local and distant metastatic disease, vascular and perineural invasion) (Goodman et al., 2009; Wein & Weber, 2005), used as outcome predictors of patients with different types of tumours, including those of head and neck region (Haddad et al., 2015).

Not only single CBC parameters like WBC, LYM, NEU or PLT counts but also N/L and P/L ratios (Bhatti et al., 2010; Kwon et al., 2012), MPV/PLT ratio (Cho et al., 2013) and adjusted PLCR for large platelets (PLCRi) have been investigated as potential diagnostic/prognostic biomarkers in human patients with both neoplastic (Bhatti et al., 2010; Kwon et al., 2012) and inflammatory conditions (Balta et al., 2013). The systemic inflammatory response, assessed by these biomarkers, has been proven to have a significant predictive survival value in human oncology patients with certain cancers, an example are ovarian cancer, non-small cell lung cancer, colorectal cancer or pancreatic ductal adenocarcinoma (Asher et al., 201; Cedres et al., 2012; Kwon et al., 2012; Smith et al., 2009).

White blood cell (WBC) count represents the total number of leukocytes (George-Gay & Parker, 2003). Leukopenia is a result of, for instance, viral infections, toxic reactions or antitumor treatments or effects of drugs suppressing the production of cells in bone marrow (Catalano, 2002). On the contrary, elevated number of

leukocytes is a frequent finding in patients with inflammatory, infectious and immune-mediated diseases (Catalano, 2002; George-Gay & Parker, 2003). In the context of neoplastic conditions, inflammation promotes tumour cell proliferation, angiogenesis and metastasis (Balkwill & Mantovani, 2001; Grivennikov et al., 2010). Negative prognostic value of leukocytosis in patients with cardiovascular diseases (Cannon et al., 2001; Salehi et al., 2013), leukaemia (McKee, 1985; Sakka et al., 2006) and solid tumours, like lung carcinoma or malignant melanoma, has been confirmed (Kasuga et al., 2001; Schniewind et al., 2005).

Neutrophils are first circulating phagocytic cells and are crucial elements of innate immune responses responsible for eliminating invading pathogens (De Larco et al., 2004). The production and modes of action of neutrophils are strictly balanced (Summers et al., 2010). In cases of inflammatory conditions, inflammatory mediators stimulate their migration to the blood where they phagocytose harmful pathogens (Summers et al., 2010). The half-life time of neutrophils is only approximately 6-8 hours when they are destructed by reticuloendothelial system (Goldman & Prabhakar, 1996; Summers et al., 2010). Neutrophilia is most commonly found in patients with acute bacterial infections while neutropenia occurs more often in cases of severe and prolonged infections, which are causing bone marrow suppression (Catalano, 2002). Neutrophils participate in the biology of tumours where they exhibit not only pro- but also antitumor functions (Sagiv et al., 2015). Neutrophils in peripheral blood or in the tumour microenvironment were shown to produce VEGF (Kusumanto et al., 2003), an important pro-angiogenic factor able to stimulate and induce angiogenesis, a process crucial for establishing a tumour microenvironment and further promotion of development and progression of tumours (Sagiv et al., 2015). Neutrophils activate immunosuppression through the inhibition of the activity of natural killer cells and activated T cells by releasing the mediators (for instance nitric oxide or reactive oxygen species) (De Larco et al., 2004). Neutrophilia correlated with poorer therapeutic outcome in human oncology patients with certain types of tumours (Donskov, 2013; Schmidt et al., 2005). On the other side, neutrophils exhibit also anti-tumorigenic effects related to the release of

antimicrobial and cytotoxic granules that have the potential to eliminate malignant cells (Brandau et al., 2012; Georgy & Houghton, 2011; Fredlender and Albeida, 2012). Suggestions of different populations of neutrophils with diverse roles in inflammatory and neoplastic processes exist (Pillay et al., 2012; Sagiv et al., 2015).

The role of lymphocytes in fighting of the immune system against tumour cells is well-established (Hernberg, 1999; Hernberg et al., 2007). Patients with advanced neoplastic conditions very often exhibit lymphopenia (Ray-Coquard et al., 2009), which has been accepted as a negative prognostic marker in humans with hematologic and solid tumours (Feng et al., 2012; Ray-Coquard et al., 2009, Schmidt et al., 2007).

The balance between pro- and antitumor immune activities is determined in the neutrophil to lymphocyte ratio (N/L), which is considered as a biomarker of systemic inflammatory and immune responses (Kim et al., 2014; Mutz et al., 2015). A high N/L ratio is demonstrating an enhanced neutrophil response (dominant pro-tumor activities) and/or relative lymphopenia (reduced antitumor response by lymphocytes) (Kim et al., 2014). Despite still unknown and still not completely investigated mechanisms of inflammation-promoting tumour progression and immune response suppression, several studies confirmed that elevated N/L ratio is associated with poorer treatment outcome and consequently worse prognosis in human oncology patients with different types of tumours (Gwak et al., 2007; Kee et al., 2012; Kim et al., 2014; Motomura et al., 2013; Porrata et al., 2010; Sharaiha et al., 2012; Teramukai et al., 2009; Walsh et al., 2005).

Platelets are not playing an important role only in haemostasis but attribute also to the inflammatory and wound healing processes (Anitua et al., 2004; George-Gay & Parker, 2003). Platelet parameters (PLT, MPV, P/L and MPV/PLT ratios, PLCRi) are used to assess the platelet activation potential. Circulating platelets differ in

morphology, size, density and reactivity (Brown et al., 1997; Martin et al., 1983). Metabolic and enzymatic activity of large platelets is higher in comparison with small platelets, which more easily react by releasing chemical mediators when stimulated (Bath & Butterworth, 1996; Mangalpally et al., 2010; Thompson et al., 1984). Increase in platelet count (thrombocytosis) is seen most commonly as a physiologic response to physiological stress, trauma or infection and in myeloproliferative disorders while causes for decreased platelet count (thrombocytopenia) include depressed consumption or destruction of platelets in bone marrow due to different stimuli (Bath & Butterworth, 1996; George-Gay & Parker, 2003). It is a well-known fact that a complex interplay between tumour cells and circulating platelets is having an effect on the growth of tumour cells, angiogenesis and spread of tumour cells to distant organs (Bambace & Holmes, 2011; Dvorak et al., 1995; Goubran et al., 2014; Sharma et al., 2014). Different types of tumours affecting kidneys, lungs, colon, ovaries, uterus and breasts correlate with increased number of peripheral blood platelets, very often termed as thrombocytosis which is considered as a part of a paraneoplastic syndrome, also shown to negatively impact the prognosis (Costantini et al., 1990; Engan and Hannisdal, 1990; Hernandez et al., 1992; Herndon et al., 1998; Kilincalp et al., 2014; Levin and Conley, 1964; Monreal et al., 1998; Symbas et al., 2000). Furthermore, it was shown that elevated P/L ratio, MPV and MPV/PLT ratio in human oncology patients with pulmonary, breast, colorectal, ovarian and pancreatic cancers, were associated with poor response to therapy and prognosis (Asher et al., 2011; Azab et al., 2012; Bhatti et al., 2010; Kwon et al., 2012; Raungkaewmanee et al., 2012; Seretis et al., 2012; Smith et al., 2008; Smith et al., 2009).

Red blood cell count, haematocrit and haemoglobin correlate as a decrease or increase of one of the red blood cell parameters is followed by similar reductions or increases of the others (George-Gay & Parker, 2003). Myeloproliferative diseases of the bone marrow can be the cause of primary polycythaemia while secondary polycythaemia usually describes an increase in red blood cell count that develops as a physiologic compensatory mechanism in conditions associated with reduced

delivery of oxygen such as cardiopulmonary diseases and chronic obstructive pulmonary disease (George-Gay & Parker, 2003). Decreases of red blood cells, haematocrit and haemoglobin are associated with haemodilution and anaemia (George-Gay & Parker, 2003). Other red blood cell parameters consisting of MCV, MCH and MCHC assist in classifying anaemia when decreased. Anaemia, a part of paraneoplastic syndrome, is a frequent finding in human and companion animal cancer patients, exerting a negative effect on their quality of life (Gilreath et al, 2014). Anaemia has also been identified as a negative prognostic factor in cancer patients (Hurter & Bush, 2007). Tumour cells and their biologically active products as well as suppressive drugs in oncology treatments such as chemotherapy for instance, can cause anaemia (Gilreath et al, 2014). Suppression of haematopoiesis is a result of a negative effect of cytokines or drugs on bone marrow that is not able to produce red blood cells (Dicato et al., 2010; Gilreath et al, 2014; Hurter & Bush, 2007).

4.2 Systemic levels of Tregs and lymphocyte T and B cell sets and subsets in dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis)

4.2.1 Periodontal disease

Periodontal diseases are most commonly diagnosed chronic, destructive, inflammatory/infectious diseases of the tooth supporting structures (periodontium) in both human (König et al., 2010) and veterinary medicine (Harvey, 1998). PD affects approximately 80% of dogs over 5 years of age (Harvey & Emily, 1992; Harvey, 1998). Interactions between oral bacteria *(Gram-negative aerobic and anaerobic bacteria)*, host's immune system and periodontium result in two main forms of PD, gingivitis and periodontitis (Pihlstrom et al., 2005). In gingivitis, only inflammation of the gingiva, visible as redness of the gums without periodontal attachment structures or alveolar bone loss, is present (AVDC, 2012). Periodontopathogenic bacteria, which stimulate cellular and humoral immune responses by the production of cytokines/chemokines and other inflammatory mediators, are responsible for development of periodontitis, an irreversible stage of the periodontal disease, associated with direct loss of gingival connective tissue, periodontal ligament, cementum and alveolar bone (Gabay, 2006; Kinane & Lappin, 2001; Offenbacher et al., 2008; Seymour et al., 1993). According to AVDC (American Veterinary Dental College) organisation nomenclature (AVDC, 2012) the severity of PD in dogs is classified into four stages. Healthy periodontium (PD0) is defined as a condition with no gingival inflammation and clinical evidence of periodontitis (AVDC, 2012). In stage 1 PD (*gingivitis*), only inflammation of the gingiva is present. Periodontal attachment structures are intact (AVDC, 2012). Stage 2 PD (*early periodontitis*) is associated with mild loss of periodontal attachment structure (<25%), stage 1 furcation and only mild radiographic signs of periodontitis (AVDC, 2012). Advanced stages of PD, stage 3 PD (*moderate periodontitis)* and stage 4 PD (*advanced periodontitis)* are associated with 25-50%, and >50% of periodontal attachment loss,

25-50%, and >50% of alveolar and horizontal bone loss as confirmed by dental radiography and stage 2 and 3 furcation, respectively (AVDC, 2012).

4.2.2 Systemic effects of periodontal disease

PD is not causing the inflammatory response only locally but also systemically when bacterial toxins, produced in periodontal lesions, translocate to systemic circulation (Geerts et al., 2002; Loos, 2005; Moutsopoulos & Madianos, 2006; Teng et al., 2002). The elevation of serum systemic inflammatory biomarkers has been shown in human patients with PD (Cray et al., 2012; D'Aiuto et al., 2004; Ebersole et al., 1997; Noack et al., 2001; Passoja et al., 2010). The association between human periodontitis and systemic diseases, such as cardiovascular diseases, endocrine/metabolic diseases (e.g. diabetes mellitus) or even body weight, has been confirmed by several studies (Beikler & Flemming, 2011; Buhlin et al., 2011; Colombo et al., 2012; D'Aiuto et al., 2006; Monteiro et al., 2009; Saxlin et al., 2011; Taylor and Borgnakke, 2008; Ylöstalo et al., 2008). In a study of Rawlinson et al. (2011) higher stages of PD in dogs were associated with increases in inflammatory markers and significant decrease of the majority of markers investigated was detected after periodontal treatment. Furthermore, several studies in veterinary medicine showed that PD was associated with several systemic diseases affecting cardiovascular and respiratory system, kidneys and liver (DeBowes et al., 1996; DeBowes, 1998; Glickman et al., 2009; Hoffmann & Gaengler, 1996; Pavlica et al., 2008). Although the association between PD and its systemic effects, exhibited on the heart or kidneys, for instance, has been demonstrated by several studies in both, human and veterinary medicine, the explanation regarding the exact relationship between the initiating factors involved in PD development and PD systemic effects still remain under investigation (Pejčić et al., 2011).

4.2.3 Immune responses

Innate (natural) immunity is a relatively non-specific mechanism of the immune system, which provides the first line defence against invading pathogens (Clark & Cooper, 2005.) The components of innate immunity include cellular barriers (physical and chemical) and cells such as macrophages, eosinophils, neutrophils, monocytes, mast cells and dendritic cells, which ingest antigens (Goldman & Prabhakar, 1996). Immune responses to bacteria include lysis of bacteria through the complement-mediated pathways and phagocytosis (Clark & Cooper, 2005; Goldman & Prabhakar, 1996; Janeway, 1989; Janeway, 1992; Janeway & Medzhitov, 2002).

The adaptive (acquired) or specific immunity is characterised by specificity, memory and the ability to distinguish self from non-self by its components; lymphocytes B and T, antigen-presenting cells (APC), dendritic cells, the complement system and cytokines (Clark & Cooper, 2005; Goldman & Prabhakar, 1996; Ouaguia et al., 2014). Adaptive immunity develops when microbial antigens react with appropriate receptors on macrophages and dendritic cells. Specific immune responses necessitate the activities of both cytotoxic and helper T cells as well as B cells. Cell-mediated and humoral immune responses of the adaptive immunity are mediated by cytokines, released by helper T cells, which mediate the activity of other cells (for instance NK cells and phagocytes) (Clark & Cooper, 2005; Cooper & Alder, 2006; Goldman & Prabhakar, 1996: Tizard, 2012).

4.2.3.1 Lymphocytes B

Lymphocytes B form in the bone marrow and are primarily responsible for establishment of humoral or antibody-mediated immune responses. Lymphocytes B with their cell surface antibody molecules bind to specific antigens, stimulating the

activation of a signaling cascade, which leads to the transcription of gens and production of antibodies against antigens. The antibodies against antigens react with an antigen that caused the initial activation of B cells. Primary B cell immune response to antigens develops when B cells first encounter an antigen and the antigen attaches to receptor. Secondary immune response develops after the primary immune response when B cells encounter the antigen again. After memory B cells recognise the antigen they multiplicate and become plasma cells which further produce antibodies (Goldman & Prabhakar, 1996; Liu et al., 1995; Pascual et al., 1994).

4.2.3.2 Lymphocytes T

While the origin of T cells are stem cells in bone marrow, the multiplication and differentiation of lymphocytes T occurs in thymus. Lymphocytes T represent 70-80% of lymphocytes in blood and lymphatic tissues and are responsible for establishment and regulation of the immune responses to antigens. Mature thymic lymphocytes T express CD3 and TCR molecules and are distinguished by two different glycoproteins $CD4^+$ and $CD8^+$ that bind to different epitopes that are a part of MHC classes I, and II molecules, participating in the development of T cells (Goldman & Prabhakar, 1996). Mature lymphocytes T differ according to different heterodimers of TCR (T cell receptor) that bind and recognise protein antigen determinants by MHC molecules. $CD8^+$ T cells bind to MHC class I while $CD4^+$ T cell bind to MHC class II (Goldman & Prabhakar, 1996).

Helper T cells ($CD4^+$) participate in the modulation of immune responses by binding of antigens to APC and B cells stimulating cell-mediated and antibody-mediated immunity, respectively. They become activated when $CD4^+$ T cells bind to antigen presented by APC or B cells on class II MHC. Once activated, these cells proliferate and differentiate to different effector T cell subsets like Th1, Th2 and Th17 cells which all have an important role in immunity as they are able to produce specific

cytokines, which facilitate different types of immune responses. Well known cytokines released by, for instance, Th1 cells are INF-γ (essential for activation of macrophages, NK-cells and $CD8^+$ T cells), interleukins (IL-2, IL-1β, IL-12) and TNF-α (Goldman & Prabhakar, 1996; Mosmann & Coffman, 1989; Mosmann & Sad, 1996).

Cytotoxic T cells, ($CD8^+$ T cells), which express the glycoprotein CD8 at their surface are produced in thymus. $CD8^+$ T cells recognise infected cells presented by MHC class I molecules on their surfaces. $CD8^+$ T cells modulate the immune responses when an infection is caused by viruses or bacteria or in tumours. Inactivation of infected or tumorous cells occurs through production and release of cytotoxic granules or production of cytokines. CD8 T cells can be inactivated through IL-10 or molecules secreted by regulatory T cells (Tregs) to an anergic state, which prevents development of autoimmune diseases (Goldman & Prabhakar, 1996; Wong & Pamer, 2003).

4.2.3.3 Central and peripheral tolerance

Immune tolerance is defined as unresponsiveness of the immune system to substances or tissues provoking negative immune responses (Goldman & Prabhaker, 1996; Murphy, 2012). Deficiencies in immune tolerance lead to the development of autoimmune reactions that can be prevented by two mechanisms, central and peripheral tolerance (Goldman & Prabhaker, 1996; MacKay, 2001).

Central tolerance eliminates and inactivates self-reactive lymphocytes T in thymus and lymphocytes B in bone marrow (Goldman & Prabhaker, 1996; Murphy, 2012) by clonal deletion or clonal anergy before they enter into periphery where they

mature and become immunocompetent cells (Goldman & Prabhaker, 1996; Hogquist et al., 2005; MacKay, 2001; Murphy, 2012).

Peripheral or extrathymic tolerance develops after maturation and entrance of T and B cells into peripheral tissues and lymph nodes (Murphy, 2012). If chemical signals, necessary for activation of T and B cells are missing, circulating lymphocytes are not able to respond. Peripheral tolerance prevents the activation of self-reactive lymphocytes by the activity of specific T suppressor cells, ignorance and anergy (Goldman & Prabhaker, 1996; MacKay, 2001). Peripheral tolerance is maintained also by Tregs, which inhibit helper and cytotoxic T cell activation by self-antigens (Goldman & Prabhaker, 1996; Murphy, 2012).

4.2.4 Regulatory T cells (suppressor T cells; Tregs)

Regulatory T cells (Tregs), firstly described by Gershon and Kondo in 1970, belong to a group of T cells with their main role in maintaining peripheral tolerance (Gershon & Kondo, 1970; Sakaguchi et al., 2009; Wing & Sakaguchi, 2010). Tregs suppress the autoreactive T cells, reduce inflammation, induce tolerance and modulate the immune response of the host (Maloy & Powrie, 2001). By suppression or downregulation of induction and proliferation of immune cells including $CD4^+$ and $CD8^+$ T cells, APC and lymphocytes B, Tregs maintain tolerance to self-antigens and protect the host from development of autoimmune diseases (Sakaguchi et al., 1995; Sakaguchi et al., 2006; Song et al., 2010). Abnormalities in the frequency and function of Tregs in controlling the immune pathologies have been demonstrated in inflammatory, infectious, (auto)-immune, allergic, and neoplastic diseases in both humans and animals (Belkaid & Rouse, 2005; Beyer and Schutze, 2006; Biller et al., 2010; Cavassani et al., 2006; Chavele and Einherstein, 2011; Cools et al., 2007; Costantino et al., 2008; Horiuchi et al., 2009; Horiuchi et al., 2010; Majlessi et al.,

2008; Mills, 2004; Nouri-Aria & Durham, 2008; Piccirillo, 2008; Robertson & Hasenkrug, 2006; Vial et al., 2009).

The activity of Tregs occurs through multiple steps including encountering and recognition of antigens on an APC and through their TCR binding of the complex to the MHC complex (Ougauia et al., 2014). The largest and the most commonly investigated group of Tregs are $CD4^+$ T regulatory T cells, divided into natural and induced Tregs (Ouaguia et al., 2014). Positive and negative selection in thymus leads to the development of natural Tregs while inducible Tregs develop at the periphery from conventional $CD4^+$ T cells (Hori et al., 2003; Sakaguchi et al., 2009; Seibel et al., 2010; Workman et al., 2009).

Natural Tregs represent only 5-10% of peripheral $CD4^+$ T cells (Workman et al., 2009). The first identification of T cells with regulatory activity correlated with the expression of CD25, α chain IL-2 receptor (Sakaguchi et al., 1995). The necessity of Tregs in controlling the physiological immune responses *in vivo* was demonstrated by Asano et al. (1996), who showed that thymectomy of mice on day 3 of life eliminates $CD4^+CD25^+$ T cell subset and leads to the development of severe lethal autoimmune diseases which diminish with $CD4^+CD25^+$ T cells replacement (Asano et al., 1996). As it has been shown that also other… "conventional T cells express CD25 when activated by T cell receptor (TCR) ligation"… (Workman et al., 2009), new markers for the identification of Tregs were suggested. FOXP3, CD25 (Workman et al., 2009) and cytoplasmatic markers like CTLA4 (co-inhibitory receptor cytotoxic T lymphocyte antigen 4) are the most common biomarkers of Tregs (Ouaguia et al., 2014). Other markers, for instance, GITR, PD-1, LAG3, HLA-DR, CD45RA/CD45RO, CD62L, CD44, CD28, CCR7, CXCR4, OX40 (CD234), folate receptor and CD39 have also been used to identify Tregs as well (Baecher-Allan et al., 2006; Beissert et al., 2006; Bolacchi et al., 2006; Deaglio et al., 2007; Shimizu et al., 2002).

“Induced or adaptive Tregs are divided into three categories including T regulatory type 1 cells (Tr1), T-helper 3 cells (Th3) and IL-35 producing $CD4^+$ T cells (iTR35)” (Bluestone and Abbas, 2003; Ouaguia et al., 2014). “Type 1 regulatory T cells (Tr1) are anergic *in vitro* and they suppress the activation of effector immune cells through production of IL-10” (Ouaguia et al., 2014). Th3 produce small amounts of TGF-ß, IL-4 and IL-10 and have been shown to produce also natural Tregs markers like CD25, FOXP3 and CTLA4. Th3 maintain the immunity of mucosal surfaces and also promote oral tolerance by negating autoimmune reactions by secreting TGF-ß and IL-10 (Beissert et al., 2006; Ougauia et al., 2014; Pot et al., 2011; Roncarolo et al., 2006).

Other T cell populations including $CD8^+$ and $CD3^+CD4^-CD8^-$ Tregs, natural killer Tregs generated in the thymus ($CD4^+$, $CD8^+$, $CD4^-CD8^-$ or $CD4^+CD8^+$) and gamma delta Tregs have been demonstrated to exhibit regulatory roles, but their role has not been completely established yet (Beissert et al., 2006; Strober et al., 1996).

4.2.4.1 FOXP3 in Tregs

The development and pathophysiological role of Tregs depend on the availability of forkhead family transcription factor (FOXP3) (Feuerer et al., 2009; Pinheiro et al., 2011). FOXP3 is expressed in both thymic-derived natural Tregs and in certain peripherally induced Tregs with suppressive function. Reduction in Tregs expressing CD4, CD25 and FOXP3 leads to development of autoimmune-mediated diseases (Buckner et al., 2010). The necessity of FOXP3, for both development and function of Tregs, was confirmed by several studies indicating that mutations in the gene encoding FOXP3 are fatal for humans and mice (Bennet et al., 2001; Chatila et al., 2000; Ziegler, 2006) resulting in the development autoimmune disease IPEX (*Immune Dysregulation Polyendocrinopathy Enteropathy X-linked syndrome*) in humans (Bennet et al., 2001; Chatila et al., 2000) and »scurfy«, a disease similar to

IPEX in mice (Brunkow et al., 2001; Ramsdell and Ziegler, 2014; Walker et al., 2003).

FOXP3 is responsible for the peripheral maintenance of natural Treg phenotype stability. "The FOXP3 protein is a transcriptional repressor of nuclear factor of activated T-cells (NFAT) and nuclear factor-kappa B (NFκB), which leads to the suppression of IL-2 secretion" (Tenorio et al., 2009). The FOXP3 protein has 431 amino acids conferring a molecular weight of 49-55 kDa. Repressor, zinc finger (ZF), leucine zipper (LZ) and forkhead (FKH) DNA represent four potential binding domains. N-terminal region of FOXP3 (repressor domain represses NFAT-mediated transcriptional activity (Bettelli et al., 2005; Lopes et al., 2006). Dimerization and suppressive function of FOXP3 in T cells are impaired by the mutant LZ domain while the FKH domain enables DNA binding and nuclear localization and is most frequently targeted in immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients (Ziegler, 2006).

4.2.4.2 Functional activity of Tregs

Natural $CD4^+CD25^+$ Tregs have an important role in controlling physiological immune responses by suppressing the autoreactive lymphocytes through contact-dependent (tight cell-cell contacts) and contact independent pathways (production of soluble immunosuppressive molecules) (Vignali et al., 2008; Workman et al., 2009). Cell to cell contacts occur in the thymus (Grossi et al., 1991), …"where Tregs are selected by peptides presented by antigen presenting cells (APC)"…. (Workman et al., 2009). The proliferation of lymphocytes reacting against self occurs through TCR (T cell antigen receptor) and other co-stimulatory cytokines (IL-10, TGF-β) and molecules (CTLA-4) through which antigen-induced Tregs regulate immune homoeostasis (Flavell et al., 2010; Nakamura et al., 2001; Sakaguchi, 2006; Shevach, 2006; Sledzinska et al., 2013). The importance of other co-stimulatory molecules

(CD28, CD80/86 CD40) for their development and function was demonstrated to be crucial as well as mice deficient in these molecules had reduced numbers of Tregs with impaired suppressive activity (Workman et al., 2009; Salomon et al., 2000).

The importance of IL-2 in the activity of Tregs was confirmed in studies on murine models and humans where neutralisation of IL-2 reduced the number of thymic and peripheral natural Tregs and caused multiorgan autoimmunity (Malek et al., 2014; Sakaguchi et al., 2001; Thornton & Schevach, 1998; Workman et al., 2009). The proliferation of natural Tregs allows specific recognition of self-antigens with concurrent stimulation of co-stimulatory molecule CD28 that supports the survival of natural Tregs by enhancing the production of IL-2 by conventional T cells (Lerman et al., 2004; Tang et al., 2003). TGF-β is another key factor in suppressing the immune responses driven by Tregs (Bluestone & Abbas, 2003; Tanq et al., 2004). TGF-β signaling in peripheral natural Tregs maintains FOXP3 expression and suppression of other cells like Th1, $CD8^+$ and NK cells (Workman et al., 2009). TGF-β was demonstrated to be crucial also for the development and suppressive activity of induced Tregs (Tr1 and Th3) (Workman et al., 2009). The development of natural and induced Tregs differs despite similarities in their phenotype and requirements for their suppressive activity (Workman et al., 2009). The development of naturally arising Tregs occurs in the thymus while induced Tregs develop in the periphery as a consequence of a weak stimulation of TCR and other exogenous antigens (Workman et al., 2009).

4.2.4.3 Tregs in animals

Regulatory T cells in animals have been investigated in several species including companion, domestic and wild animals under normal and pathological conditions (Allers et al., 2010; Benham et al., 2009; Biller et al., 2007; Bolzer et al., 2009; Ellis & Demartini, 1985; Garden et al., 2011; Gerner et al., 2010; Hamza et al., 2012; Joshi et al., 2004; Junginger et al., 2012; Kaser et al., 2008; Keppel et al., 2008;

Lankford et al., 2008; Manigold et al., 2006; Mcneilly et al., 2010; Mexas et al., 2008; Mitra et al., 2010; Pinheiro et al., 2011; Porter et al., 2007; Seibel et al., 2010; Seo et al., 2009; Silva-Campa et al., 2009; Singh et al., 2009; Vahlenkamp et al., 2004; Wongyanin et al., 2010).

Regulatory T cells in dogs were firstly mentioned by Weiden et al. (1976), who showed, that suppressor population of cells might be involved in preventing the development of GVHD (graft-versus-host disease). Detection of Tregs in dogs is possible by using specific monoclonal antibodies targeted against rodent (mouse/rat) FOXP3 and specific antibodies against canine CD25 (Abrams et al., 2010; Biller et al., 2007; Junginger et al., 2012; Mizuno et al., 2009; Pinheiro et al., 2011; Rissetto et al., 2010). Due to their crucial role in controlling the immune responses, Tregs have been investigated in peripheral blood, lymph nodes and tumour tissues in dogs with inflammatory, neoplastic and infectious diseases (Biller et al., 2007; Biller et al., 2010; Horiuchi et al., 2009; Horiuchi et al., 2010; Keppel et al., 2008; O'Neill et al., 2009; Tominaga et al., 2010; Veenhof et al., 2010; Veenhof et al., 2011). The regulatory function of these cells has been described also *in vitro* (Abrams et al., 2010; Pinheiro et al., 2011).

4.2.5 Immune responses in periodontal disease

The pathogenesis of PD is mediated by innate and adaptive immune responses (including humoral and cell-mediated immunity), provoked by periodontopathogens (Ebersole & Taubman, 1994; Okui et al., 2008). The most important risk factor of PD development with immunoregulatory function is ascribed to the T cell phenotype although B cell responses play an important role as well (Gemmell et al., 2001; Houri-Haddad et al., 2007). The role of T cell phenotype, more specifically Th1/Th2 relationship in PD is still under investigation. The debate regarding the involvement of Th1 cells in stable periodontal lesions and Th2 responses in the states of progressive PD lesions is still open (Gaffen & Hajishenegallis, 2008; Gemmell et al.,

2001). A healthy periodontium can be maintained only if the balance between different T cell subsets (e.g. $CD4^+$, $CD8^+$ T cells and Tregs) and B cells is maintained and well-controlled (Garlet et al., 2010). Different results were obtained when evaluating the frequency of T cell subsets ($CD4^+$, $CD8^+$) in periodontal lesions and/or peripheral blood of PD patients compared to healthy controls (Okada et al., 1984; Taubman et al., 1984). As both, increases and decreases in T cell subsets ($CD4^+$ and $CD8^+$) have been reported, contradictory results regarding CD4/8 ratios being, decreased or increased, have been demonstrated (Celenligil et al., 1993; Katz et al., 1988; Stoufi et. al., 1987). No correlation of CD4/8 ratio with PD has been confirmed as well (Engel et al., 1984). Syrjanen et al. (1984) indicated and increased CD4/8 ratio in the periodontal tissues of patients with aggressive PD and their families. The results of a study of Meng and Zheng (1989) revealed no significant differences in the mean ratios of $CD4^+/CD8^+$ T cells from diseased and healthy periodontal tissues, but individual differences in T cell subsets and CD4/8 ratios were found between patients with the same disease (marginal gingivitis, juvenile and adult periodontitis). Despite findings of several authors that high CD4/8 ratios correlate with pronounced inflammatory infiltrate in periodontitis lesions and lower CD4/8 ratios with PD lesions with less inflammatory cells present, the exact role and impact of helper and cytotoxic T cells in the development of early and/or advanced periodontitis lesion still remains under investigation.

Immunoregulatory role of B cells in PD includes theirs modulatory direct and/or indirect effects on other cells (Lund et al., 2005; Porakishvili et al., 2001). B cells are activated by cytokines produced predominantly by Th2 cells (Yamamoto, Fujikashi et al., 1997) or by direct contact with antigens (Gemmell et al., 2007). Activated B cells transform into plasma cells, which promote immunoglobulins that identify and bind to antigens (Berglundh et al., 2007). The adaptive immune responses, properly regulated by B cells, protect the host from negative effects of pathogens by establishing specific immunity with a memory component (Graves & Cochran, 2003).

4.2.5.1 Tregs and periodontal disease

Investigation of the role of Tregs in PD is an area of intense research activity in human medicine and mice models in the last several years. Although the development of PD is very well investigated, the pathophysiological mechanisms guiding the PD development and progression still remain to be elucidated. It has been demonstrated that osteoclastogenesis (process that relies on many factors) is mediated by Treg-derived cytokines such as TGF-β, IL-10 or IL-4 or CTLA-4 that were all shown to be present in diseased periodontal tissues (Cardoso et al. 2008; Garlet et al. 2010; Sasaki et al., 2004). Tregs are involved in maintaining the metabolic equilibrium between formation and resorption of alveolar bone (Mori et al., 2007). While the majority of studies investigating the role of Tregs in PD concluded, that increases in Tregs correlate with the pathological states of periodontium, less authours agree, that an increased frequency of Tregs (identified with phenotypic markers such as FOXP3, CTLA-4, IL-10, TGF-β, GITR, CD103 and CD45RO) is maintaining the periodontium in a healthy state (Indumathy et al., 2012; Cardoso et al., 2008; Dutzan et al., 2009; Ito et al., 2005; Nakajima et al., 2005; Okui et al., 2008). Investigations regarding the role of Tregs in patients with PD were not focused only to the measurements in the local periodontal environment but also to detection of Tregs in peripheral blood and revealed decreased number of Treg cells ($CD4^+CD25^+$) (Cardoso et al., 2008; Indumathy et al., 2012; Takeuchi et al., 1991). In contrast, periodontitis lesions, compared to healthy and gingivitis lesions, were demonstrated with increased expression of FOXP3 (Cardoso et al., 2008; Duarte et al., 2011; Kim et al., 2010; Nakajima et al., 2005; Okui et al., 2012).

4.3 Immunohistochemical expression of Ki-67 and VEGFR-2 in canine inflammatory, benign and malignant head and neck conditions

4.3.1 Physiological angiogenesis

The development and growth of blood vessels occur during embryogenesis through two related processes e.g. vasculogenesis and angiogenesis (Risau, 1997; Risau & Flamme, 1995; Olsson et al., 2006). Vasculogenesis is the formation of blood vessels by the *in situ* development of cells of endothelium from angioblasts while angiogenesis refers to the formation and occurrence of new blood vessels from the pre-existing vessels (Risau, 1997; Risau & Flamme, 1995). After formation of blood vessels by vasculogenesis, angiogenesis becomes the predominant mechanism by which blood vessels form, both during the later stages of embryonic development and throughout adulthood (Coffin & Poole, 1988). A balance between proangiogenic and anti-angiogenic factors in physiological angiogenesis maintains normal and vital processes such as growth and development of an organism and wound healing (Armulik et al., 2005; Coffin & Poole, 1988; Risau, 1997; Risau & Flamme, 1995).

4.3.2 Pathological angiogenesis

Pathological angiogenesis in chronic inflammatory and neoplastic diseases, in both human and veterinary medicine, occurs due to disregulations in angiogenesis, which lead to an increased formation of new blood vessels (Fava et al., 1994). Angiogenesis, providing oxygen and nutrients differs in the context of neoplastic disease development. The growth of tumours (restricted to few cubic millimetres) is limited in the absence of angiogenesis while pronounced pathological angiogenesis is associated with an increased growth of tumours and/or development of metastases (Folkman, 1990; Folkman, 1995) as demonstrated in solid tumours affecting breast,

colon, lung, kidney and bladder (Albo et al., 1994; Bosari et al., 1992; Denijn & Ruiter, 1993; Macchiarini et al., 1992; Toi et al., 1993; Weidner et al., 1993).

4.3.3 Vascular endothelial growth factors (VEGFs)

4.3.3.1 Physiological and pathological role of VEGFs

Physiological vasculogenesis and physiological and pathological angiogenesis are guided by growth factors restricted to vascular endothelium (Carmeliet, 2003). VEGFs participate in the early phases of embryonic development, more specifically, when the development of the cardiovascular and central nervous system and the formation of bones occurs and in the angiogenesis processes, e.g. formation of blood vessels throughout life including wound healing and ovulation (Brock & Lee, 2002; Carmeliet et al., 1996; Ferrara et al., 2003; Ferrara & Davis-Smyth, 1997). Exerting its important physiological role in above-mentioned processes, VEGFs overexpression in tumours correlated with increased angiogenesis as well as proliferative and metastatic potential of tumour cells (Brock & Lee, 2002; Folkman, 1995).

The VEGF family consists of growth factors A, B, C, D and platelet derived growth factor, classified as glycoproteins of a molecular weight of approximately 40 kDa (Olsson et al., 2006). Alternative splicing of VEGF factors leads to the formation of different VEGF isotypes with diverse biological activities (Neufeld et al., 1999). Binding of VEGFs to its receptors, VEGFR-1 (Flt-1), VEGFR-2 (FLK-1/KDR), VEGFR-3 (Flt-4) or other co-receptors, such as heparin sulphate proteoglycans and neuropilins, is responsible for their activation and function (Olsson et al., 2006). VEGF-A binds to VEGFR-1 and VEGFR-2 and thus plays an important role in vasculogenesis and angiogenesis processes. Proteolytic reactions regulate the activity

of VEGF family members enabling specific interactions with receptors, which bind to these factors (Olsson et al., 2006). Regulatory role of VEGFs is similar to other receptor tyrosine kinases (RTKs), such as PDGFRs and EGFRs (Olsson et al., 2006). Their activity correlates with dimerization of the tyrosine kinases, as well with the formation of docking sites for signal transducers (Olsson et al., 2006). Cytokines such as FGF-4, PDGF, TNF-α, TGF-β, IL-1β or IL-6, hormones (gonadotropins) and oncogenes such as the activated forms of Src and Ras have an impact on the expression of VEGFs (Detmar et al., 1994; Mukhopadhyay et al., 1995; Rak et al, 1995).

4.3.3.2 Hypoxia and angiogenesis

Although the production of VEGF depends on the local oxygen concentration (Papetti & Herman, 2002), hypoxia has been considered as one of the major stimulators of VEGFs expression (Damert et al., 1997; Minchenko et al., 1994; Neufeld et al., 1999). This was confirmed in several studies, indicating an increased expression of VEGFs is present in hypoxic tumours (Collet et al., 2014) where hypoxia helps to initiate proliferation, angiogenesis and death by stimulating the activity of transcription factors, for instance, inducible factors of hypoxia (HIFs) (Harris, 2002; Lee et al., 2009). The transcriptional activity of HIFs occurs through VEGF (Hanahan, 1997; Papetti & Herman, 2002; Pugh and Ratcliffe, 2003; Shweiki et al., 1992) that bind to *cis* elements in the VEGF promoter, stimulating the release of pro-angiogenic factors (Forsythe et al., 1996; Papetti & Herman, 2002; Wang & Semenza, 1993). The formation and growth of new blood vessels, stimulating the growth of a tumour, is a consequence of VEGFs binding to vascular endothelial growth factors receptors (VEGFRs) (Collet et al., 2014; Ferrara, 1999). Expression and/or overexpression of VEGFs correlate with resistance to therapy and poor therapeutic outcome in patients with certain types of tumours (Chai et al., 2014; Folkman, 2007).

4.3.3.3 Mechanisms of tumour angiogenesis

Tumour angiogenesis, arising from avascular or vascular tumours, differs. Hypoxic areas within avascular tumours stimulate expression of transcription factors sensing hypoxia, such as the HIFs (Wang & Semenza, 1993) which in turn induce accumulation of pro-angiogenic proteins, such as VEGF, FGF and IL-8 stimulating angiogenesis (Forsythe et al., 1996; Stinga et al., 2011). In vascular tumours, the tumour cells, inducing the expression of angiopoietin-2 in the pre-existing vessels, cause regression of the part of the tumour mass due to endothelial cell apoptosis. As vessels regress, the tumour becomes avascular. According to the presence of hypoxia, upregulation of angiogenic factors induces the production of new blood vessel supply (Papetti & Herman, 2002).

4.3.4 Vascular endothelial growth factor receptors (VEGFRs)

VEGFRs belong to the receptor tyrosine kinase (RTK) family and have similar structure as PDGF family (Olsson et al., 2006). Receptors, VEGFR-1 and VEGFR-2, consist of an extracellular domain with 7 immunoglobulin-like loops and an intracellular domain, important for tyrosine kinase activity (Halper, 2010). Despite a high percentage (80%) of homology among the Ig-like domains of the VEGFRs, the heterogeneity of the rest 20% in their structure contributes to differential ligand binding, kinase activity and biological activity of receptors (Koch et al., 2011; Singh et al., 2005). Immunoglobulin domain-3 in VEGFR-2 is important in determination of ligand-binding specificity (Olsson et al., 2006).

The function and activity of VEGF/VEGFRs is exhibited primarily in vascular system where they are primarily expressed although VEGFR expression occurs also

in non-endothelial cells. VEGFR-1 and VEGFR-2 are localised to both vascular and lymphatic endothelial cells while VEGFR-3 is primarily present in lymphatic endothelium (Nilson et al., 2004; Olsson et al., 2006).

Binding of ligands of VEGF to VEGFRs induces dimerization (homodimerization and heterodimerization) of the receptors and activation of RTK activities, two processes, leading to autophosphorylation of the receptors (Olsson et al., 2006; Takahashi et al., 2001). Phosphorylated receptors further recruit interacting proteins resulting in the activation of signalling pathways exhibiting both physiological and pathological effects. Hypoxia-inducible factors (HIFs) regulate the expression of VEGFR-1 by binding to promoter region of VEGFR-A (Olsson et al., 2006). Increased expression of HIF-α has been shown as a negative prognostic biomarker in patients with oropharyngeal SCCs (Aebershold et al., 2001; Semenza, 2004). The activity of VEGFR-2 is regulated both negatively and positively. Trimeric G proteins guide a positive regulation while negative regulation occurs through the phosphotyrosine phosphatases Src-homology phosphatase-1 and 2 (Olsson et al., 2006).

VEGFRs have been demonstrated to be involved in both physiological and pathological vascular endothelial cell biology (Senger et al., 1993). VEGFR-2 is, among other receptors, the major and the most important receptor responsible for endothelial cell function since early beginning of the embryonic endothelial development (Shalaby et al., 1995). The importance of VEGFR-2 (Flk-1) at the physiological level was proved by Shalaby et al. (1995) who demonstrated that homozygous embryos deficient in VEGFR-2 die because of defects in the haematopoietic and endothelial cell development in very early stages of embryonic development, that is, 8.5-9.5 days post-coitum.

Tumour cells produce various pro-angiogenic factors, including VEGF and its receptors in both humans and animals (Bergers & Benjamin, 2003; Folkman, 2007; Rebuzzi et al., 2007; Restucci et al., 2004). VEGFR-2 is considered as the earliest biomarker for endothelial development and the primary driver of pathological processes associated with neovascularization, for instance, tumours, as other VEGFRs are also expressed on a wide variety of other non-endothelial cells (Shalaby et al., 1995; Shibuya & Claesson-Welsh, 2006). The overexpression of VEGFR-2, with paracrine or autocrine activation, is one of commonly detected dysregulations of receptor tyrosine kinases in cancer, although somatic mutations, involving deletions and point mutations, exist as well (Walter et al., 2002). Due to deprivation of nutrients and oxygen supply, especially in the centre of a tumour, tumour cells, in order to survive, stimulate production of VEGF, which further, with co-operation of VEGFRs, trigger angiogenesis processes (Neufeld et al., 1999) affecting proliferative, migrative and survival potential of endothelial cells and thus the growth of tumours (Fukumura et al., 2001; Holmqvist et al., 2003; Innocenti et al., 2003; Koch et al., 2011; Senger et al., 1983; Stetler-Stevenson, 2008; Takahashi et al., 2001). In addition, formation of new blood vessels involving VEGF/VEGFRs pathways, with an impact on the progression of tumours, correlated also with inactivation of tumour suppressor genes (e.g. p53) or down-regulation of angiogenesis factors like inhibitor thrombospondin 1 (TSP1) (Dameron et al., 1994; Rastinejad et al., 1989).

4.3.5 Targeting VEGFs/VEGFRs pathways

Anti-VEGF/VEGFR drugs block pathological angiogenesis processes with targeting VEGF/VEGFR signalling pathways and have been demonstrated to have a therapeutic benefit for different types of tumours overexpressing VEGF and/or VEGFRs in both human and veterinary medicine (Bergers & Hanahan, 2008; London et al., 2012; Hahn et al., 2008; Hurwitz et al., 2004; Peterson et al., 2004; Thamm et al., 2012; Yu et al., 2002). In veterinary medicine, two tyrosine kinase

inhibitors as monotherapy or as a part of multimodal treatment approach are used. Masitinib inhibits c-kit and also PDGFR alpha and beta, Lyn, FGFR3 and FAK pathways while toceranib blocks VEGFR-2, PDGFR, Kit and Flt-3 (Hahn et al., 2008; Thamm et al., 2012). Ozao-Choy et al. (2009) showed in an *in vivo* animal cancer model, that these drugs, despite having cytotoxic potential, exhibit immunomodulatory effects on certain immune cells like myeloid-derived suppressor cells and Tregs.

4.3.6 Cell proliferation

Cell proliferation, representing a balance between cell division and death, is a physiological process responsible for the growth and maintenance of homoeostasis in almost all tissues and under many circumstances (Pitiyage, 2009). In cancer, where mutations or deletions of certain genes cause the disruption of the balance between cell proliferation (division of cells) and apoptosis (programmed cell death), uncontrolled proliferation of tumour cells leads the development of benign and/or malignant tumours (Bishop, 1987; Tumuluri et al., 2002; Van Diest et al., 1998;). Proliferation activity of tumour cells is assessed by proliferating cell nuclear antigen (PCNA) and Ki-67 antigen (Gerdes et al., 1983). Ki-67 antigen was firstly described by Gerdes et al. (1983) who demonstrated the potential use of Ki-67 for the evaluation of proliferating cells in tumours. Ki-67 is a biomarker of the growth fraction of cells as it is expressed in all active parts of the cell cycle (G1, S, G2 and mitosis) but not in resting cells (G0) (Gerdes et al., 1983; Gerdes et al., 1986). The antibody, most commonly used for the detection of Ki-67 in both human and veterinary medicine, is monoclonal antibody, MIB-1 (Bergin et al., 2011; Couture et al., 2002; Fischer et al., 2011; Gerdes et al., 1983; Pena et al., 1998; Webster et al., 2007). In human head and neck cancer contradictory results regarding the prognostic value of Ki-67 might relate to the analysis of a combination of various head and neck cancers of different anatomical locations, histological types, biological activity and clinical behaviour. Analysis of more homogenous groups of tumours, arising from

the head and neck, indicated, that the cell proliferative activity, estimated by Ki-67, is a reliable prognostic factor (Pich et al., 2004). Other tumour cell death related markers, like p21 along with Ki-67, appeared as strong negative prognostic factor in HNSCC as well (Fischer et al., 2011).

The role of Ki-67, an important radioresistance factor, has been assessed in several studies, where radiotherapy directed to the cell cycle, was used for the treatment of neoplasia affecting head and neck (Budach et al., 2006; Couture et al., 2002; Marcu, 2013). Tumour hypoxia is considered as a negative prognostic factor with a negative impact on the therapeutic outcome as well as overall survival of patients with tumours of the head and neck (Bittner & Grosu, 2013; Swartz et al., 2015). In hypoxic environment, an important radioresistance factor, cells are preferentially in G0 phase (Hall et al., 1994). According to several authors, the tumour cells of head and neck tumours with low proliferative activity indicate hypoxia and radioresistance (Couture et al., 2002; Freudelsperger et al., 2012; Kennedy et al., 1997). Couture et al. (2002) reported significantly higher incidence of local recurrences of oral cavity carcinoma tumours of patients treated with radical course of radiotherapy compared to patients with low proliferative activity of tumour cells (Ki-67 < 20%).

4.4 Accelerated chemoradiotherapy protocol for advanced canine head and neck squamous cell carcinoma

4.4.1 Human head and neck squamous cell carcinoma (HNSCC)

Several histological types of tumours affect head and neck with squamous cell carcinoma being one of the most frequently diagnosed neoplasms of this anatomical site (Dobrossy, 2005; Marcu, 2013; Reuter et al., 2007; Rogers et al., 2005). HNSCC tumours arise from oral cavity, oropharynx, hypopharynx, larynx, sino-nasal and nasal region and can be treated effectively only if diagnosed early enough (Vokes et al., 1993).

Several aetiology factors (e.g. tobacco smoking, alcohol consumption, papillomavirus infection, oral leukoplakia, atmospheric pollution and poor dental hygiene) have been mentioned as initiating factors of HNSCC (Dobrossy, 2005; Hashibe et al., 2007; Zhang et al., 2000).

The choice of treatment strategy for HNSCC depends on the primary site of the tumour (location) and the staging results, more specifically, the presence/absence of regional lymph node or distant site metastases (Kramer et al., 2005; Pignon et al., 2000; Reuter et al., 2007; Rogers et al., 2005). Surgery and radiotherapy exhibit similar effectiveness for small HNSCC while more advanced HNSCC require multimodal treatment approach, consisting of surgical resection, if possible, with adjuvant treatments consisting of radiotherapy and chemotherapy and/or biologically target drugs (Pignon et al., 2000).

Location of the tumour and staging results of patients with HN tumours impact the prognosis (Brana & Siu, 2012). The best prognosis is achieved in patients with early-stage HNSCC who are treated with surgery and/or radiotherapy while the prognosis for patients with an advanced stage of the disease is considered to be poor (Reuter et al., 2007; Seiwert et al., 2007; Shibuya et al., 2002). The expected survival of 5 years, for patients with advanced stage tumours, is less than 10%, if treated with radiotherapy alone (Rogers et al., 2005; Reuter et al., 2007). These patients have an increased risk to develop recurrences loco-regionally and/or distant site metastases and this fact reduces the overall survival even more considerably and is approximately 6 to 9 months (Kirby et al., 2006; Reuter et al., 2007). Despite significant improvements made in the multimodality treatment approach of patients with unresectable HNSCC, including alterations in the fractionation radiotherapy protocols, integrating radiotherapy with chemotherapy and adding biologically targeted drugs, the treatment strategies for advanced HNSCC are still not standardised.

4.4.2 Radiation therapy and altered fractionation radiotherapy protocols

Human patients with unresectable HNSCC are treated with radiation therapy, which is delivered alone or concurrently with chemotherapy (Brana and Siu, 1012). Traditional radiotherapy protocols involve radiation of patients with HNSCC once daily using fractions of 1.8 to 2 Gy (to a total dose of approximately 70 Gy) in a period of 6 to 7 weeks (Seiwert et al., 2007). Altered fractionation protocols, including hyperfractionated and accelerated radiotherapy protocols, have been used as well, but are still under investigation (Ang et al., 1990; Corvo, 2007; Fu et al., 1995; Gwozdz et al., 1997; Mendenhall et al., 2000; Million et al., 1985; Overgaard et al., 2003; Wang, 1988). Hyperfractionation or accelerated fractionation radiotherapy protocols improved the therapeutic outcome and positively impacted the disease-free and overall survival of patients with HNSCC in comparison with

conventional radiotherapy protocols (Bourhis, Overgaard et al., 2006; Bourhis, Lapeyere et al., 2006; Fu et al., 2000; Hliniak et al., 2002; Horiot et al., 1992; Jackson et al., 1997; Overgaard et al., 2003; Pinto et al., 1991; Poulsen et al., 2001; Skladowski et al., 2000).

4.4.3 Altered radiotherapy fractionation protocols and concurrent chemo-radiotherapy protocols

Several clinical studies demonstrated the beneficial effect of combining altered fractionation radiotherapy protocols, such as accelerated or hyperfractionated radiotherapy with chemotherapy on the overall survival of patients with these types of tumours (Ang, 1988; Budach et al., 2006; Bourhis, Overgaard et al., 2006; Bourhis, Lapeyere et al., 2006). According to the meta-analysis of Budach et al. (2006), addition of chemotherapy, to any radiation therapy protocol, extends survival to 12 months and is at 2 years 13% to 15% and at 5 years approximately 8%. In comparison, the overall survival benefit at 5 years, of patients treated with altered fractionation protocols, accelerated or hyperfractionated, when compared to conventional radiotherapy protocols with no chemotherapy, was, according to the meta-analysis conducted by Bouhris, Overggard et al. (2006), 3.4%. An interesting finding was that patients, treated with an accelerated radiotherapy without chemotherapy, showed no significant overall survival benefit when compared to the overall survival of patients treated with conventional radiotherapy protocols while hyperfractionation increased median survival for approximately 14 months (Bouhris, Lapeyere et al., 2006). The benefit of accelerated fractionation was demonstrated as well and was according to Bouhris, Overgaard et al. (2006) approximately 2% at 5 years. Altered fractionation radiotherapy caused significantly lower percentage (23% reduction in risk) of loco-regional failures with the absolute reported benefit of 8.5% at 5 years (Bourhis, Overgaard et al., 2006). Although the results of Budach and Bourhis meta-analyses revealed the positive impact of hyperfractionated or accelerated radiotherapy protocols on the treatment outcome and as well on the

overall survival of patients with HNSCC, the role of chemotherapy, applied as a radiosensitizer or concomitantly with radiotherapy, is still unclear and needs to be investigated further (Budach et al., 2006; Bourhis, Overgaard et al., 2006; Bourhis, Lapeyere et al., 2006).

4.4.4 Canine squamous cell carcinoma of the head and neck

Canine tumours arising from head and neck are heterogeneous group of neoplasia affecting hard and soft tissues of the dog's oral cavity (mandible, maxilla), lingual, sublingual and tonsillar region, nasal cavity, frontal sinuses and nasal planum (Liptak and Withrow, 2007; McEntee, 2012; Withrow, 2007).

Squamous cell carcinoma (SCC) is one of the second most frequent oral malignancies detected although other malignant tumours affecting the oral cavity including malignant melanoma, sarcomas (fibrosarcoma, osteosarcoma, multilobular osteochondrosarcoma, hemangiosarcoma), undifferentiated malignant tumour of young dogs, extramedullary plasmacytoma, oral lymphoma, neuroendocrine cell tumours, mast cell tumour and granular cell myoblastoma can be diagnosed (Liptak and Withrow, 2007; MacEwen et al., 1977; Madewell et al., 1976; McEntee, 2012; Wilson and Dungworth, 2002).

Squamous cell carcinomas also affect lips and cheek although other types of tumours such as fibrosarcoma, mast cell tumour, soft tissue sarcoma and malignant melanoma might be diagnosed in these anatomical locations as well. The most common tumour identified with predilection to lingual and sublingual lesions is SCC but other tumour types such as malignant melanoma, mast cell tumour, fibrosarcoma, adenocarcinoma, hemangiosarcoma, rhabdomyoma and rhabdomyosarcoma might develop as well. Carcinomas (adenocarcinoma, SCC, mast cell tumour) are the most commonly

diagnosed salivary gland tumours in dogs (Liptak and Withrow, 2007; McEntee, 2012).

Two-thirds of intranasal cavity tumours are carcinomas, consisting of adenocarcinoma, SCC and undifferentiated carcinoma tumours. These are followed by sarcomas (fibrosarcoma, osteosarcoma, chondrosarcoma and undifferentiated sarcoma), lymphoma, mast cell tumour and hemangiosarcoma. The majority of frontal sinus tumours are squamous cell carcinomas. The same is true for canine nasal planum tumours, although other types such as lymphoma, fibrosarcoma, haemangioma, malignant melanoma, mast cell tumour, fibroma and histiocytoma have been reported (Withrow, 2007).

Malignant head and neck tumours are locally aggressive and remain a therapeutic challenge, especially when presented at an advanced stage (Liptak and Withrow, 2007; McEntee, 2012; Withrow, 2007). Caudal oral cavity, tonsillar and lingual SCC, like other oral tumours, for instance, malignant melanomas, exhibit higher metastatic potential in comparison with rostrally located SCC (Liptak and Withrow, 2007; McEntee, 2012; Modiano et al., 1999). The metastatic potential for nasal cavity or sino-nasal tumours, at the time of the presentation, is considered low, but metastases can develop in the late course of the disease with regional lymph nodes and lungs being the most commonly affected sites (Patnaik et al., 1984; Patnaik, 1989).

Treatment options and strategies depend on several factors, with the most important factors being the type of the tumour and staging results, patient's overall health and the client's preferences. Radiation therapy is typically recommended as an adjuvant treatment for inoperable or incompletely excised oral and tonsillar SCC while for canine patients with nasal cavity or sino-nasal tumours radiotherapy remains the only treatment possibility (Liptak and Withrow, 2007; McEntee, 2012; Withrow, 2007).

Curative intent radiotherapy protocols, for squamous cell carcinomas affecting head and neck in dogs, consist of fractions of 2.7 Gy to 4.2 Gy, delivered daily or on alternate days, with a total dose ranging from 48 to 57 Gy (Bateman et al., 1994; Blackwood and Dobson, 1996; Freeman et al., 2003; Proulx et al., 2003; Theon et al., 1997). The lowest rates of local tumour recurrence and best survival times are described for dogs with early stage oral SCC treated with surgery and/or radiotherapy. The prognosis remains poor, especially for advanced stage canine head and neck SCC (Liptak and Withrow, 2007; Withrow, 2007).

Although the long-term prognosis for advanced canine nasal cavity or sino-nasal tumours is guarded to poor, radiation therapy has a positive effect on the overall survival (Evans et al., 1989; Patnaik et al., 1984; Turek and Lana, 2007; Wilson and Dungworth, 2002). Curative intent radiotherapy treatment protocols (full course or definitive radiation) are considered the most effective of achieving local control and result in median survival generally in the 12-month range (ranging from 8–19.7 months) with the 1- and 2- year survival rates ranging from 43-60 % at 1 year and 11-44 % at 2 years (Adams et al., 1987; Adams et al., 1998; Adams et al., 2005; Lana et al., 1997; McEntee et al., 1991; Nadeau et al., 2004; Theon et al., 1993). They cause moderate to severe acute side effects, which can be prevented with supportive medical care but can temporarily compromise the quality of life. Palliative intent radiotherapy protocols can be also used as they have been shown to improve clinical signs and survival, compared to palliative care only (Gieger et al., 2008; Mellanby et al., 2002; Morris et al., 1994; Kleiter et al., 2004; Rassnick et al., 2006).

In order to obtain a better loco-regional tumour control and better overall treatment outcome of dogs with nasal cavity or sino-nasal tumours variations in radiotherapy protocols including accelerated radiotherapy, boost technique and the use of radiosensitizers have been investigated (Adams et al., 2005; Lana et al., 1997; LeBlanc et al., 2004; Nadeau et al., 2004; Thrall et al., 1993). The growth of tumours was improved as well as overall times, although not with all techniques. As the acute

reactions, as well as increased late side effects, occurred with these protocols the approaches are not an accepted treatment modality for these types of tumours in dogs. The causes for non-responsiveness of canine nasal and/or sino-nasal tumours to radiotherapy may include rapid tumour cell proliferation and the presence of tumour hypoxia within the tumour volume, which are all factors of radioresistance (LaDue et al., 1999). Considering the specific tumour biology of SCCs and results of conventionally fractionated radiotherapy, modified radiation fractionation protocols, with an aim to improve the therapeutic outcome of patients suffering from advanced stages of HNSCC have been tried. Accelerated radiotherapy protocols, alone or postoperatively, have already been used for the treatment of canine intranasal tumours, but not oral and tonsillar SCC tumours, and resulted in median survival of 19.7 and 47.7 months, respectively (Adams et al., 1998; Adams et al., 2009; Liptak and Withrow, 2007). Unfortunately, accelerated, coarsely fractionated definitive protocols resulted in severe late side effects in 40% of dogs and are not established radiotherapy treatment modality in veterinary medicine (Adams et al., 1998).

Investigation of multiple pathways in the pathobiology of tumours affecting head and neck is complex and extensive but will definitely help to optimise and potentially individualise the treatment approach for patients affected with this types of tumours not only in human (Harari, 2005) but also in veterinary medicine.

5 MATERIALS AND METHODS

5.1 Complete blood count parameters and indices in canine inflammatory and neoplastic head and neck conditions

5.1.1 Patients

The medical records of 236 dogs were reviewed. 71 clinically healthy dogs, 73 dogs with stage 3,4 PD (moderate to advanced periodontitis) and 92 dogs with neoplastic head and neck conditions (31 dogs with carcinomas, 19 dogs with melanomas, 24 dogs with sarcomas, 18 dogs with epulides) who had undergone medical examination and received treatment at Animal Hospital Postojna, Slovenia from September 2011 to January 2014 were included. The CBC of the investigated groups of dogs were retrospectively retrieved and reviewed from the database of the Animal Hospital Postojna, Slovenia. A group of dogs defined to be healthy dogs was included to compare the investigated parameters as controls. These dogs were presented to Animal Hospital Postojna, Slovenia for elective surgical procedures (ovariectomy, castration) or other diagnostic non-invasive procedures (x-rays of hips etc.) and all had a routine blood analysis (CBC) performed as a part of a complete pre-anaesthetic check-up. All dogs were otherwise clinically healthy with no concurrent systemic diseases detected.

5.1.2 Staging of the canine periodontal disease

The teeth in the oral cavity of dogs affected with stage 1 (gingivitis) and stage 3,4 PD (moderate to advanced periodontitis) were prior periodontal prophylaxis and periodontal treatments evaluated by periodontal examination including evaluation of the presence of teeth, measurement of the probing depth, determining the periodontal

attachment loss and furcation exposures and assessment of teeth mobility and dental radiography.

5.1.3 Staging and treatment of the canine neoplastic head and neck conditions

Dogs with histologically confirmed spontaneous head and neck neoplasia were reviewed. The pre-treatment evaluation included a complete physical examination, a CBC, serum biochemistry profile and urinalysis and CT of the head and neck region and/or thorax for staging and treatment planning purposes. For evaluation of potential metastatic spread, regional lymph nodes were evaluated with fine needle aspiration or biopsy. Clinical stage for oral/pharyngeal head neck tumours was classified according to the tumour dimension (T) (T1< 2cm; T2 2-4 cm; T3 > 4cm) without (substage a) or with (substage b) evidence of bone involvement, regional lymph node status (N) and presence or absence of distant metastases (M) using World Health Organization (WHO) Tumour Node Metastasis system for classification of tumours in domestic animals (Owen, 1980). Modified Adams staging scheme (Adams et al., 2009) was used for sino-nasal tumours and those were classified as stage 1 (unilateral, no bone involvement), stage 2 (bone involvement detected but no evidence of the extension of tumour into soft tissues), stage 3 (extension of tumour into orbit) and stage 4 (evidence of cribriform lysis).

All dogs with NHNC were treated with curative intent multimodality treatment protocols. Patients with oral/pharyngeal neoplasia were treated with surgical resection + non-steroidal anti-inflammatory drugs (NSAIDs) or surgical resection + NSAIDs + radiotherapy or radiotherapy + NSAIDs. The treatment response was assessed according to Nguyen et al. (2015) treatment response criteria based on physical examination and/or diagnostic imaging results (radiography and/or CT) and was defined as a complete response (CR; complete tumour regression), partial response (PR; at least 30% reduction of the tumour), stable disease (SD; less than 30% reduction or 20% increase of the tumour) and progressive disease (PD; the

appearance of new lesions or at least a 20% increase of the tumour). Overall survival (OS) was the time of the initial diagnosis to the time of death from any cause or the date the patient was last known to be alive.

5.1.4 Analysis of blood samples and calculation of complete blood count indices

The complete blood count (CBC) parameters of whole blood, collected in ethylene-diamine-tetra-acetic acid (EDTA) anticoagulant, were measured with a haematology analyser (Sysmex pocH-100iV, Kobe, Japan) in Animal Hospital Postojna, Slovenia. The blood analysis was performed as a part of the general health check-up, usually at the time of the presentation, before performing any diagnostic steps or treatments. N/L and P/L ratios were calculated as ratios of the neutrophils and platelets to lymphocytes, respectively. MPV/PLT ratio was calculated as a ratio between MPV to PLT. Adjusted P/L ratio for large platelets (PLCRi) was obtained by multiplying the platelet-large cell ratio (PLCR) with calculated P/L ratio.

5.1.5 Statistical analysis

Statistically significant differences between the groups investigated (healthy control dogs (HD); dogs with stage 3,4 PD (moderate to advanced periodontitis) and dogs with neoplastic head and neck conditions (NHNC)) were preliminary explored by Kruskal-Wallis test. Multivariable analysis were performed according to the polytomous logistic regression model and linear regression model in the case of predicting overall survival in a group of dogs with malignant head and neck tumours. The analysis was performed using R software (R Core Team, 2013) with the net package (Venables & Ripley, 2002) for polytomous logistic regression. P values <0.05 were considered as statistically significant.

5.2 Systemic levels of Tregs and other lymphocyte T and B cell sets and subsets in dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis)

5.2.1 Patients

Mature mixed breed, small to medium size dogs (weighing < 20kg) presented for the treatment of stage 1 PD (gingivitis) and stage 3,4 PD (moderate to advanced periodontitis) between September 2013 and September 2014 were included in the study. The exclusion criteria for dogs included professional dental treatment performed in the last 3 years and evidence of any concurrent systemic disease. The latter was also confirmed by a complete clinical examination with CBC, serum biochemistry and urinalysis in all cases to detect any possible contraindications before induction to general anaesthesia. The owners gave the consent to both, the required treatment and the use of obtained peripheral blood and regional lymph node aspirates for haematological and flow cytometric analysis. No owners declined inclusion of their dogs in the study.

Peripheral blood samples for complete blood count and flow cytometric analysis were obtained before sedation and induction to general anaesthesia while fine needle aspirates of palpable and/or enlarged mandibular lymph nodes, for cytological and flow cytometric evaluation, were obtained immediately after induction to general anaesthesia as a part of general periodontal oral cavity examination.

A mixture of medetomidine and methadone was used for sedation. General anaesthesia was induced using propofol IV. Following endotracheal intubation the dogs were maintained with isofluorane or sevofluorane in a mixture of oxygen/air

(0.5L/0.5L) using a non-rebreathing (dogs weighing < 5kg) or rebreathing system (dogs weighing > 5kg) circuit.

The periodontal examination under general anaesthesia included assessment of the tooth presence, probing depth, periodontal attachment loss, furcation exposure and tooth mobility and dental radiography in both groups of dogs investigated. Periodontal treatment included supragingival and subgingival scaling and polishing of teeth in dogs with stage 1 PD (gingivitis) and in dogs witn stage 3,4 PD (moderate to advanced periodontitis) followed by extraction of periodontally affected teeth in a group of dogs with stage 3,4 PD (moderate to advanced periodontitis). Teeth there were mobile and diagnosed with furcation exposure and/or horizontal or vertical bone loss of more than 25% of the periodontal attachment of one or more tooth roots were extracted.

5.2.2 Flow cytometric analysis

The peripheral blood samples for flow cytometric analysis were obtained into appropriate anticoagulant 3ml vacutainer tubes (EDTA) while fine needle aspirates of the regional lymph nodes were placed into vacutainers with 0.5ml RPMI 1640 (RPMI; Roswell Park Memorial Institute) containing 5% fetal bovine serum (FBS) and 0.2% sodium azide.

Cell integrity and antigenic expression was maintained by analysis of peripheral blood samples as well as aspirates of the regional lymph nodes within 24 hours from the collection of samples.

Before immunostaining procedure the samples were retained at temperature 20-25°C. The cellularity of a sample was asessed with a haematology analyser. Indirect (using unconjugated or biotin-conjugated monoclonal or polyclonal antibodies recognising cell surface antigens) and direct FC immunostaining (for intracellular antigens with permeabilisation step before staining) can be performed.

Indirect FC staining (according to the manufacturer's instructions) was performed with PBS/BSA, PBS and erytrolyse red blood cell lysing buffer. Firstly, lysis of erythrocytes in blood samples was performed with erythrocyte lysis buffer (2ml) in a plastic tube with the incubation of the sample for 10 minutes at room temperature when the sample was centrifuged and the supernatant decanted. Next step was dilution of a sample with phosphate buffered saline (PBS; pH 7.4) in a conical tube in the ratio of 1:1 (500 µl of each) adjusting the cell suspension to a concentration of 1 x 10^6 cells/ml with PBS/BSA buffer. 100 µl of the cell suspension was then aliquoted into the tubes required for the analysis. Primary antibodies (Table 1) were added at reccomneded dilutions, the samples mixed well and incubated at 4°C for 30 minutes. Cells were then washed with 2ml of PBS/BSA, centrifuged at 400g for 5 minutes and the supernatant discarded. One million cells in a volume of 100µl were used for immunostaining. Positive controls were cells incubated with a fluorescent antibody directed to an epitope while negative controls were controls using secondary reagents of appropriate isotype matched controls. A secondary reagent was added at recommended dilution, the sample mixed well and incubated at 4°C, avoiding light, for 30 minutes. After that, the sample was centrifuged at 400g for 5 minutes and the supernatant discarded. Cells were resuspended in 200µl of PBS or with 200µl of 0.5% paraformaldehyde in PBS when required. The analysis of samples was performed according to the manufacturer's instructions within 24-48 hours after staining and fixation.

Intracellular staining of FOXP3 was performed with a cross-reactive murine PE-conjugated FOXP3 antibody (FOXP3 staining set, clone FJK-16s, eBioscience) with

permeabilization and fixation/permeabilization buffer used according to the manufacturer's instructions. Cells were firstly resuspended in fixation/permeabilization buffers overnight at 4°C. Cells were washed twice (with permeabilization buffer) and incubated with anti-mouse/rat FOXP3 antibody (1µl per $1x10^6$ cells) diluted in FACS buffer for 30 minutes at 4°C. Cells were additionally washed twice in permeabilization buffer followed by resuspension in FACS buffer. A directly conjugated rat IgG2A antibody was used as the isotype control.

Samples were analysed with a flow cytometer emitting light at 488 nm and with excitation and emission wavelengths for FC fluorochromes FITC (525 nm) and PE (575 nm). The results were analysed with Cell Quest Software and were expressed as dot, density, contour plots and histograms (Figure 1).

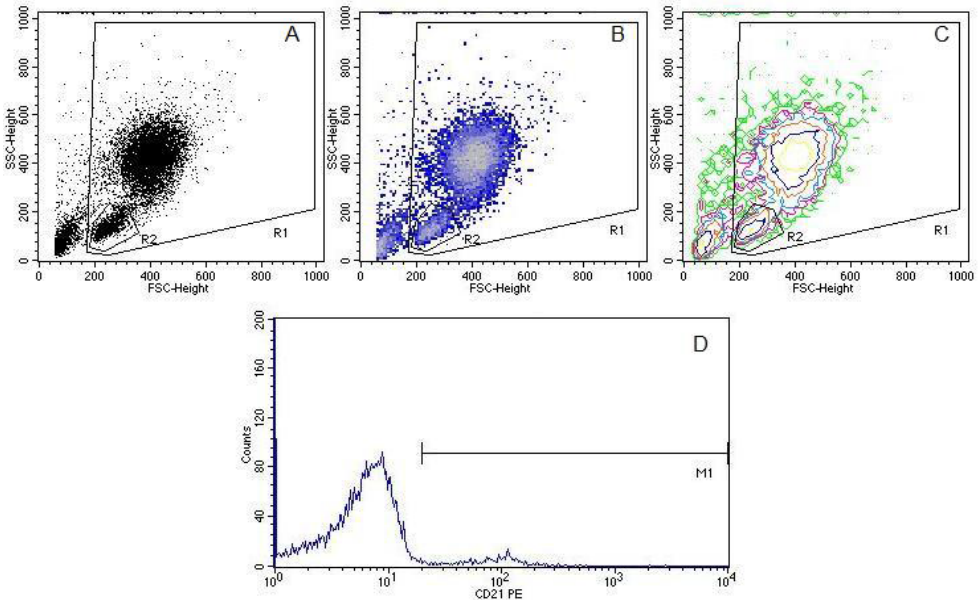


Figure 1: Flow cytometric analysis of peripheral blood sample of a healthy dog (Martini, 2013, pp 10).

Figure 1 is representing the FC results presented as dot (A), density (B) and contour plots (C) and histogram (D). Forward (FSC) and side (SSC) scatter presented in panel A, B and C indicate how separation of cells passing through the beam distinguish different populations of cells. FSC correlates with the size of the cells while SSC depends on the density. Larger and more granular cells produce a large population with high SCC and FSC. Monocytes are large but not so granular and produce high FSC but lower SCC then granulocytes. Lymphocytes produce a separate population with less FSC and as they are not so granular also have low SSC. While R1 gate indicates excluded debris the gate R2 is a gate including only small-sized cells. Picture D is a histogram indicating the fluorescence after staining with anti-CD21 antibody where only a few cells (B-lymphocytes) stained positive (M1) (Martini, 2013, pp 10).

5.2.3 Statistical analysis

Statistical differences in the parameters investigated (the absolute numbers of WBC and LYM and the relative numbers of LYM and T and B lymphocyte populations and subpopulations ($CD4^+$, $CD8^+$, $CD5^+$ T-cell subsets and $CD21^+$ B cells) and Tregs ($CD4^+CD25^+$, $CD4^+CD25^+FOXP3^+$) between dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis) were compared by Mann-Whitney U test (Bonferroni adjustment, p-values <0.005). The analysis was performed using R software (R Core Team, 2013).

Associations between the immunophenotype markers investigated were assessed using Paerson's and Sparman′s rank correlation method tests. Correlation coefficients were interpreted according to Cohen's criteria (Cohen, 1988): $r = 0$, no correlation; $0 < r < 0.1$, trivial correlation; $0.1 \leq r < 0.3$, slight correlation; $0.3 \leq r < 0.5$, moderate correlation; $0.5 \leq r < 0.7$, substantial or high correlation; and $r \geq 0.7$ very high correlation. $p<0.05$ was considered to indicate a statistically significant difference.

Table 1: Antibody panel used for immunophenotyping of canine peripheral blood and regional lymph node samples by flow cytometry.

Nr. of tube	CD molecule	Ab	Specificity	Ab clone	Source
1	$CD5^+$-FITC	RatIgG2a	T lymphocytes	YKIX332.3	Serotec
2	$CD21^+$-PE	Mouse IgG1	B lymphocytes	CA21D6	Serotec
3	$CD8^+$-PE	Rat IgG1	T cytotoxic	YCATE55.9	Serotec
4	$CD4^+$-FITC	Rat IgG1	T helper	YKIX302.9	Serotec
5	$CD25^+$-PE	Mouse IgG1	Tregs	P4A10	Serotec
6	$FOXP3^+$-FITC	Rat IgG1	Tregs	FJK-16s	Bioscience

5.3 Immunohistochemical expression of Ki-67 and VEGFR-2 in canine inflammatory, benign and malignant head and neck conditions

5.3.1 Tissue samples

Tissue samples of inflammatory, benign and malignant canine head and neck conditions were collected from the clinical cases presented to Animal Hospital Postojna, Slovenia where the cases were diagnosed and treated. A total of 54 head and neck samples were identified. All the biopsy and surgical samples were histopathologically and immunohistochemically analysed, archived and retrieved from the Veterinary Pathology Department of Comparative Biomedicine and Food Science of Veterinary Medicine of the University of Padua, Italy. There were 9 stomatitis samples, 11 benign samples consisting of 6 samples of acantomatous epulis and 5 fibromatous epulis samples, 17 epithelial tumours (10 oral SCC and 7 sino-nasal SCC) and 17 other samples consisting of 7 malignant melanoma and 10 oral sarcoma samples. The medical records of dogs were reviewed and data regarding the clinico-pathological parameters and survival were collected for the analysis.

5.3.2 Immunohistochemical analyses for Ki-67 and VEGFR-2

Immunohistochemical analysis of samples consisted of steps including formalin fixation of excised tissue samples, sectioning of tissue blocks (4 μm thick sections), deparaffinisation in alcohol (xylene) and dehydration in different concentrations of alcohol solutions for 5 minutes of each. 100% xylene and solutions of 100 %, 90 %, 70 %, 50 % alcohol and finally distilated water were used. 0.3% hydrogen peroxide-methanol solution was used for the blockage of endogenous peroxidase, which was performed for 20 minutes at room temperature. Further step was retrieval of an

antigen, which was performed with 10mM citrate buffer (pH 6.0) for 2 minutes at 121°C and washing with phosphate buffered saline (PBS) for 5 minutes. 5% bovine albumin or 10% goat serum in phosphate buffer saline (PBS) for 20 minutes at 42°C was used for nonspecific protein binding. Monoclonal mouse anti-human VEGFR-2 antibody (clone A3, Santa Cruz Biotechnology, Santa Cruz, INC, CA, dilution 1:50) already described for the use in veterinary medicine (Al-Dissi et al., 2007; Biller et al., 2007) and the Ki-67 antibody (clone MIB-1, Dakocytomation, UK, dilution 1:50), a mouse monoclonal anti-human antibody raised in mouse against human recombinant Ki67 peptide, were used. Primary antibodies were diluted in water and washed with PBS for 5 minutes. If already diluted in 2.5% BSA immediate incubation with primary antibody was performed. The primary antibodies (VEGFR-2 antibody and Ki-67 monoclonal antibody) were then applied to tissue slice sections, which were then incubated during the night (for 14 to 18 hours) at 4°C. After incubation, additional washes in PBS, for 5 minutes of each, were performed. Diluted (1:400) secondary antibodies (anti-mouse and anti-rabbit), depending on the primary antibody, if monoclonal or polyclonal, were applied to tissues for 20 minutes at 42°C followed by incubation with avidin-biotin-peroxidase complex (Dako EnVisionTM) at a dilution of 1:400 for 30 minutes at room temperature followed again by additional washes in PBS. The reaction was visualised by the reaction of peroxidase complexed with the secondary antibody with a solution of chromogen substrate incubated for 8 minutes using peroxidase substrate kit (DABkit) - diaminobenzidine hydrogen peroxide (Vector Laboratories). The sections were thereafter counterstained with Mayer's haematoxylin for 1 minute and washed with running water for 5 minutes. The last step was dehydration in graded alcohol solutions of 50%, 70%, 90% and 100% and xylene.

The positive control tissues for Ki-67 were formalin-fixed, paraffin-embedded sections of canine hyperplastic lymph node, basal epidermis or adnexa internal to the tissues and vessel endothelium for VEGFR-2. Negative controls were obtained by replacing the primary antibody with antibody diluent.

The first examination of the slides prepared consisted of evaluating the slides at low power (40X) to areas with high numbers of Ki-67 and VEGFR-2 positive cells. Within each area, a high power field (400X) was selected and cells were counted. Ki-67 and VEGFR-2 expression was counted as the percentage of positively labelled nuclei by counting 1,000 cells in 10 high power fields. The final result for Ki-67 and VEGFR-2 was defined as the percentage of immunoreactive tumour cells out of the total number of counted cells.

For each marker, Ki-67 and VEGFR-2, the parameters evaluated were: 1. *localization of the marker:* in the nucleus, cytoplasm and membrane; 2. *number of positive cells:* expressed in percentages; 3. *intensity of expression:* using a scoring system; negative; + mild (pale brown); ++ moderate (moderately brown); +++ strong (intense brown). An immunohistochemical (IHC) scoring system based on the percentage of Ki-67 or VEGFR-2 immunostained cells in a combination with Ki-67 or VEGFR-2 labelling intensity was used.

Intensity in staining:

negative (no staining) → 0
mild staining (+) → 1
moderate staining (++) → 2
strong staining (+++) → 3

Percentage of tumour cells staining (%):

no positive cells or the presence of < 10% positive tumour cells;
(no or low expression) → 0
10-25 % of positive cells → 1
26-50 % of positive cells → 2
> 50 % of positive cells → 3

A total IHC was obtained by multiplying the results of both variables investigated (getting the product) ranging from 0 to 9. Ki-67 and VEGFR-2 samples were considered positive if the IHC score was 1 or > 1 meaning that at least 10% or more tumour cells stained positive.

5.3.3 Statistical analyses

Data were analysed by parametric and non-parametric statistical analyses. Mann-Whitney test (Wilcoxon rank sum test) was used to detect differences in the Ki-67 and VEGFR-2 between different types of head and neck conditions investigated. Univariable statistical analysis was performed to identify the presence of potential prognostic factors. Curves for overall survival were generated by the Kaplan-Meier product limit method (log-rank, Cox model). Variables investigated included location (oral *vs* sino-nasal), histological type (epithelial *vs* mesenchymal), stage (I+II *vs* III+IV), grade (low *vs* high) and the presence of necrosis (yes *vs* no). Continuous variables (mitotic count, Ki-67 and VEGFR-2 expressing cells were dichotomized using the median value as a cut-off. Low Ki-67 and VEGFR-2 expression were defined as expression below the median level for Ki-67 and VEGFR-2. High Ki-67 and VEGFR-2 expression was defined as expression above the median level. Associations between the variables investigated (age, gender, grade, histotype, mitotic activity, Ki-67, VEGFR-2) were assessed using Sparman′s rank correlation method tests. The interpretation of correlation coefficients is the same as in a study of Al-Dissi et al. (2010). Correlation coefficients were interpreted according to Cohen's criteria (Cohen, 1988); $r = 0$, no correlation; $0 < r < 0.1$, trivial correlation; $0.1 \leq r < 0.3$, slight correlation; $0.3 \leq r < 0.5$, moderate correlation; $0.5 \leq r < 0.7$, substantial or high correlation; and $r \geq 0.7$ very high correlation. $p<0.05$ was considered to indicate a statistically significant difference.

5.4 Accelerated chemoradiotherapy protocol for the treatment of advanced canine head and neck squamous cell carcinoma

5.4.1 Patients

Seven dogs with histologically confirmed locally advanced oral SCC (n=2), tonsillar SCC with regional lymph node metastasis (n=1), sino-nasal SCC (n=3) and nasal planum SCC (n=1) and treated at the Animal Hospital of Postojna, Slovenia are presented. A thorough physical examination, the complete blood count (CBC) and the serum biochemical analysis with electrolytes were performed in all cases. Skull and dental radiographs (n=2), abdominal radiographs and ultrasound (n=7) and head and neck and thorax computed tomography (CT) (n=7) were performed for staging and radiotherapy planning purposes. The complete diagnostic work-up and chemoradiotherapy treatment was performed with dogs under general anaesthesia.

Premedication was performed using methadone (0.0125-0.05 mg/kg) and medetomidine (0.3-0.5 mg/kg) i.m. General anaesthesia was induced using i.v. propofol (1-3 mg/kg) to effect. The patients were intubated and maintained with isofuorane or sevofluorane in an oxygen/air mixture (0.5 liter/min of each) using Mapleson F anaesthetic circuit. Hemoglobin oxygen saturation, non-invasive blood pressure, electrocardiography, end tidal CO_2, heart and respiratory rates and body temperature were monitored throughout the anaesthesia. Fluid homeostasis was maintained by administration of Ringer's lactate solution (3-5 ml/kg/h) i.v.

The diagnosis was confirmed in all cases through histopathological examination of biopsies of the tumours. Tumours were staged according to the World Health Organization (WHO) Tumour Node Metastasis system for classification of tumours in domestic animals. Clinical stage for oral/tonsillar SCC tumours was classified as

T1 (<2 cm), T2 (2-4 cm) or T3 (>4 cm), and substage a (no bone invasion), or substage b (bone invasion) (Owen et al, 1980). Patients with sino-nasal tumours were classified into 1 of 4 stages according to the Adams et al. (2009) classification staging system. Stage 1 was tumour confined to one nasal passage, paranasal sinus (maxillary recess and/or caudal recess), or frontal sinus with no bone involvement. Stage 2 was tumour identified involving bone with no orbital, subcutaneous, or submucosal mass. Stage 3 was involvement of the orbit or presence of a subcutaneous or submucosal mass. Stage 4 was tumour extending into the nasopharynx or cribriform plate.

5.4.2 Radiation therapy

A nine-day accelerated radiotherapy protocol with concomitant carboplatin developed by Fidel et al. (2011) was used. Radiotherapy was performed with an external beam megavoltage radiation. Radiation was delivered with a 6 MV linear accelerator Elekta Philips 75/5 with treatments given twice daily for 7 treatment days (Monday-Friday, Monday-Tuesday) in a 9-day period using 3.5 Gy fractions for a total of 49 Gy with a minimum of 6 hours between fractions. Once the animals were anaesthetised they were placed on the treatment table in the required position (lateral or sternal) and secured to the table with adhesive tape. Treatment planning was performed manually on all cases and field sizes and treatment prescription depths were decided based on each case CT study. The clinical target volume (CTV) was defined as the visible gross tumour volume (GTV), based on each CT study, together with the ipsi-lateral regional mandibular and retropharyngeal lymph nodes and empirical normal tissue cranio-caudal and dorso-ventral margins of a minimum 1-2 cm. An additional 7-10 mm margin was added around the CTV to define the planned target volume in order to account for set up errors, geometrical uncertainties. All dogs were treated with parallel-opposed beams. The isocenter was positioned in the middle of the tumour volume in all cases. Only SAD (100cm) calculation techniques

were used. A 0.5 cm or 1 cm tissue equivalent bolus material (Superflab) was used as needed in most cases to improve the dose distribution at the surface.

Carboplatin was used as a radiosensitizer to a total dose of 300 mg/m^2 divided into four increments (each 75 mg/m^2) delivered before 1st, 4th, 8th and 13th radiotherapy. The chemotherapy was delivered as a 20-30 minutes intravenous infusion approximately 1 hour before commencing radiotherapy treatment.

Support therapy included as required antibiotics, amoxicillin clavulanic acid (20 mg/kg q 12 hrs) in 4/7 dogs, metronidazole (20 mg/kg q 12 hrs) in 3/7 dog, non-steroidal anti-inflammatory drug piroxicam (0.3 mg/kg q 24 hrs) in 1/7 dogs, meloxicam (0.1 mg/kg q 24hrs) in 3/7 dogs or carprofen (4 mg/kg q 24 hrs) in 3/7 dog and other pain medications, tramadol (1-3 mg/kg q 12 hrs) in 2/7 dogs.

5.4.3 Follow-up

Appearance of tumours and tumour response was assessed during the treatment by taking photographs for visual comparison. The first follow-up examination was performed three weeks after completion of radiotherapy when all patients were assessed for acute side effects. Follow-up information was obtained by examination of the patients at the Animal Hospital of Postojna (AHP), Slovenia, or by telephone contacts with owners or the referring veterinarians. Three-month intervals examinations including complete clinical examination and regular thoracic radiography to evaluate for distant metastasis were suggested to the owners to assess the response to therapy. At 3-6 months intervals after completing the radiotherapy a CT of the head and neck and thorax was performed in all dogs with SNSCC and in 2 dogs with oral/tonsillar SCC. For dogs that were not re-evaluated at AHP the referring veterinarians and owners were contacted by phone about general health and

tumour status and in case of death, if the cause was tumour-related or unrelated. The tumour response was determined through physical examination and/or radiography or CT and was defined, according to the Nguyen et al. (2015) response evaluation criteria for solid tumours in dogs and determined as a complete response (CR; complete regression of a tumour), partial response (PR; at least 30% reduction in the tumour), stable disease (SD; less than 30% reduction or 20% increase in the tumour) and progressive disease (PD; the appearance of new lesions or at least a 20% increase in the tumour). Responders were defined as dogs with either CR or PR.

The time (days) from the first radiation therapy treatment to the time of the local recurrence of a tumour and/or appearance of distant metastatic disease was defined as time to progression (TTP) while the overall survival time (OS) was determining the time (days) from the radiotherapy commencement to the date of a patient's death or the date of the last follow-up performed or the date of the data analysis for animals still alive.

5.4.4 Toxicity assessment

Radiation toxicities, evaluating the acute and late radiation effects, were assessed during radiation therapy and according to the examinations performed and/or owner's reports monthly. Veterinary radiation treatment oncology group (VRTOG) morbidity-scoring scheme for both early and late effects was used to score the radiation toxicity effects (LaDue & Klein, 2001) and Veterinary Cooperative Oncology Group Common Terminology for Adverse Events (VCOG-CTAE v1, 2011) was used for grading the chemotherapy toxicities.

6 RESULTS

6.1 Complete blood count parameters and indices in canine inflammatory and neoplastic head and neck conditions

6.1.1 Univariate analysis of complete blood count parameters and indices (N/L, P/L and MPV/PLT ratios, PLCRi) of healthy dogs, dogs with stage 3,4 PD (moderate to advanced periodontitis) and dogs with neoplastic head and neck conditions

Table 2 *Patients profile*

Variable	*HD (n=71)*	*Stage 3,4 PD (n=73)*	*NHNC (n=92)*	*P*
WBC	9.19 (3.89 - 16.4)	8.3 (3.9 - 21.5)	11.15 (4.5 - 28.9)	<0.001
RBC	6.67 (4.38 - 8.8)	7.07 (4.38 - 8.85)	6.63 (4.25 - 9.4)	0.009
HGB	15.6 (11 - 19.6)	15.5 (9 - 20)	14.25 (8.4 - 24.5)	<0.001
HCT	0.45 (0.32 - 0.55)	0.45 (0.27 - 0.56)	0.42 (0.26 - 0.58)	<0.001
MCV	66.8 (59.6 - 91.6)	64.1 (57.3 - 91.6)	62.7 (51.7 - 70.2)	<0.001
MCH	23.1 (21.2 - 33.1)	22.5 (11.1 - 33.1)	21.8 (1.4 - 24.7)	<0.001
MCHC	35 (31.5 - 37.2)	34.9 (31.9 - 37.1)	34.7 (20.7 - 36.7)	0.097
PLT	330 (202 - 446)	354 (135 - 655)	342 (108 - 767)	0.034
LYM	2.3 (1 - 3.87)	1.6 (0.7 - 2.8)	1.25 (0.3 - 3.4)	<0.001
NEU	6.4 (2.35 - 11.52)	6.3 (2.5 - 16.7)	9.1 (3.5 - 24.9)	<0.001
EOS	0.34 (0.12 - 1.4)	0.5 (0.1 - 2.7)	0.75 (0.16 - 2.3)	<0.001
RDW-SD	34.2 (30.6 - 53.1)	35.2 (30.5 - 52.1)	35.7 (31 - 46.7)	<0.001
RDW-CV	12.6 (10.4 - 24.5)	12.2 (9.8 - 37.1)	12.95 (10.1 - 22)	0.001
PDW	13 (8.5 - 17.6)	12.2 (7.4 - 23.8)	12.25 (9 - 22.2)	0.170
MPV	6.3 (5.1 - 13.1)	10.9 (7.6 - 13.3)	10.4 (0.2 - 20.6)	<0.001
P-LCR	32.6 (12.7 - 53.7)	31.7 (8.2 - 55.5)	29.55 (0.43 - 63.5)	0.560
N/L	2.7 (1.61 - 4.43)	4.09 (1.18 - 11.3)	8.59 (1.86 - 30.7)	<0.001
P/L	145.31 (73 - 315.3)	224 (75 - 696)	290.5 (62.13 - 1051)	<0.001
MPV/PLT	2.2 (1.28 - 5.99)	2.9 (1.5 - 9.26)	3.04 (0.08 - 9.26)	<0.001
P-LCRi	47.41 (15.24 - 98.9)	69.18 (25.23 - 145.4)	90.04 (9.46 - 221.01)	<0.001

*Kruskal-Wallis test; Values represent median values.

Legend; HD: healthy dogs; PD: dogs with periodontal disease; NHNC: dogs with neoplastic head and neck conditions; WBC: white blood cells, RBC: red blood cells; HGB: haemoglobin, HCT: haematocrit, MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration, PLT: platelet count, LYM: lymphocyte count, NEU: neutrophil count, EOS: eosinophil count, RDW-SD: red cell distribution width standard deviation, RDW-CV: red cell distribution width expressed as coefficient of variation; PDW: platelet distributed width, MPV: mean platelet volume, P-LCR: platelet large cell ratio, N/L: neutrophil to lymphocyte ratio, P/L: platelet to lymphocyte ratio, MPV/PLT: mean platelet volume to platelet count ratio; P-LCRi: adjusted platelet to lymphocyte ratio for large platelets

The results of the parameters investigated were preliminary explored by univariate statistical analysis (Kruskal-Wallis test) and revealed significant differences in the CBC parameters (WBC, HGB, HCT, MCV, MCH, LYM, NEU, RDW-SD, MPV) and indices (N/L ratio, P/L ratio, MPV/PLT ratio, PLCRi) when comparing healthy controls, dogs with stage 3,4 PD (moderate to advanced periodontitis) and dogs with NHNC ($p<0.05$). The N/L ratio of healthy dogs was 2.7, of dogs with stage 3,4 PD (moderate to advanced periodontitis) 4.09 and of dogs with NHNC 8.59. The N/L index of dogs with NHNC was approximately 3 times higher in comparison with healthy controls and 2 times higher in comparison with dogs affected with stage 3,4 PD (moderate to advanced periodontitis). Healthy controls exhibited the lowest P/L ratio of 145.31 while P/L ratio of diseased dogs was detected to be higher with stage 3,4 PD (moderate to advanced periodontitis) affected dogs having a ratio of 224 and dogs with NHNC diagnosed with the highest P/L ratio of 290.5. MPV/PLT ratio of healthy dogs was 2.2. Dogs with stage 3,4 PD (moderate to advanced periodontitis) and dogs with NHNC exhibited similar values of MPV/PLT ratio (2.9 and 3.04 respectively). PLCRi was the lowest in healthy dogs (47.41) and the highest in dogs with NHNC (90.04). Dogs with stage 3,4 PD (moderate to advanced periodontitis) had the PLCRi of 69.18. No significant differences in the MCHC, PLT, PDW and P-LCR parameters were detected. The significant differences in RBC, RDW-SD and RDW-CV between groups were detected. Despite significant differences detected the values were still in the reference ranges.

6.1.2 Multivariate analysis of complete blood count indices (N/L, P/L and MPV/PLT ratios, PLCRi) of healthy dogs, stage 3,4 PD (moderate to advanced periodontitis) and dogs with neoplastic head and neck conditions

Table 3: *Multivariate analysis of CBC indices (N/L, P/L and MPV/PLT ratios, PLCRi) between healthy dogs, stage 3,4 PD (moderate to advanced periodontitis) and dogs with neoplastic head and neck conditions*

Variable	*PD vs. HD*		*NHNC vs. HD*		*NHNC vs.PD*	
	OR (95 % CI)	*P*	*OR (95 % CI)*	*P*	*OR (95 % CI)*	*P*
N/L	1.48 (0.98 - 2.23)	0.061	2.27 (1.49 - 3.47)	<0.001	1.54 (1.29 - 1.82)	<0.001
P/L	1.02 (1.01 -1.03)	<0.001	1.01 (1.01 - 1.02)	<0.001	0.99 (0.99 - 1.00)	0.006
MPV/PLT	2.58 (1.67 - 3.97)	<0.001	2.10 (1.28 - 3.46)	0.004	0.82 (0.56 - 1.19)	0.298
P-LCRi	1.00 (0.98 - 1.02)	0.804	1.02 (1.00 - 1.04)	0.039	1.02 (1.01 - 1.03)	0.005

Table 3 represents the results of polytomous logistic regression analysis of CBC indices (N/L, P/L, MPV/PLT ratios and PLCRi) revealing significant differences of CBC indices (N/L, P/L and MPV/PLT ratios and PLCRi) between groups of dogs investigated.

N/L ratio

Although dogs with 3,4 PD (moderate to advanced periodontitis) exhibited higher N/L ratio in comparison with healthy controls the difference was not significant ($p=0.061$) but the values was close to statistical significance. Significant difference in N/L ratio between healthy controls and dogs with NHNC was confirmed ($p<0.001$). Significant differences in N/L ratio between dogs with stage 3,4 PD (moderate to advanced periodontitis) *vs* dogs with NHNC were also detected with dogs affected with stage 3,4 PD (moderate to advanced periodontitis) exhibiting significantly lower

N/L ratio in comparison with dogs with NHNC which exhibited the highest N/L ratio among all groups of dogs investigated ($p<0.001$).

P/L ratio

According to multivariate statistical analysis P/L ratio was significantly different between groups of dogs investigated. Both groups of dogs with inflammatory and NHNC exhibited significantly higher P/L ratio in comparison with healthy controls ($p<0.001$). The significant difference was detected also when comparing dogs with stage 3,4 PD (moderate to advanced periodontitis) and dogs with NHNC but the significance was less pronounced ($p<0.006$).

MPV/PLT

The ratio between MPV and PLT (MPV/PLT) was significantly different when comparing dogs with stage 3,4 PD (moderate to advanced periodontitis) and NHNC with healthy controls ($p<0.001$; $p=0.004$, respectively). Due to similar MPV/PLT ratios of dogs with moderate to advanced periodontitis (stage 3 to 4 PD) and NHNC no significant differences between groups were detected.

PLCRi

Less significant differences between groups of dogs investigated were found for PLCRi. No significant differences in PLCRi between healthy control dogs and dogs with stage 3,4 PD (moderate to advanced periodontitis) were determined ($p=0.804$) When comparing dogs with NHNC with healthy controls and dogs with stage 3,4 PD (moderate to advanced periodontitis) significant differences were found ($p=0.039$, $p=0.005$, respectively).

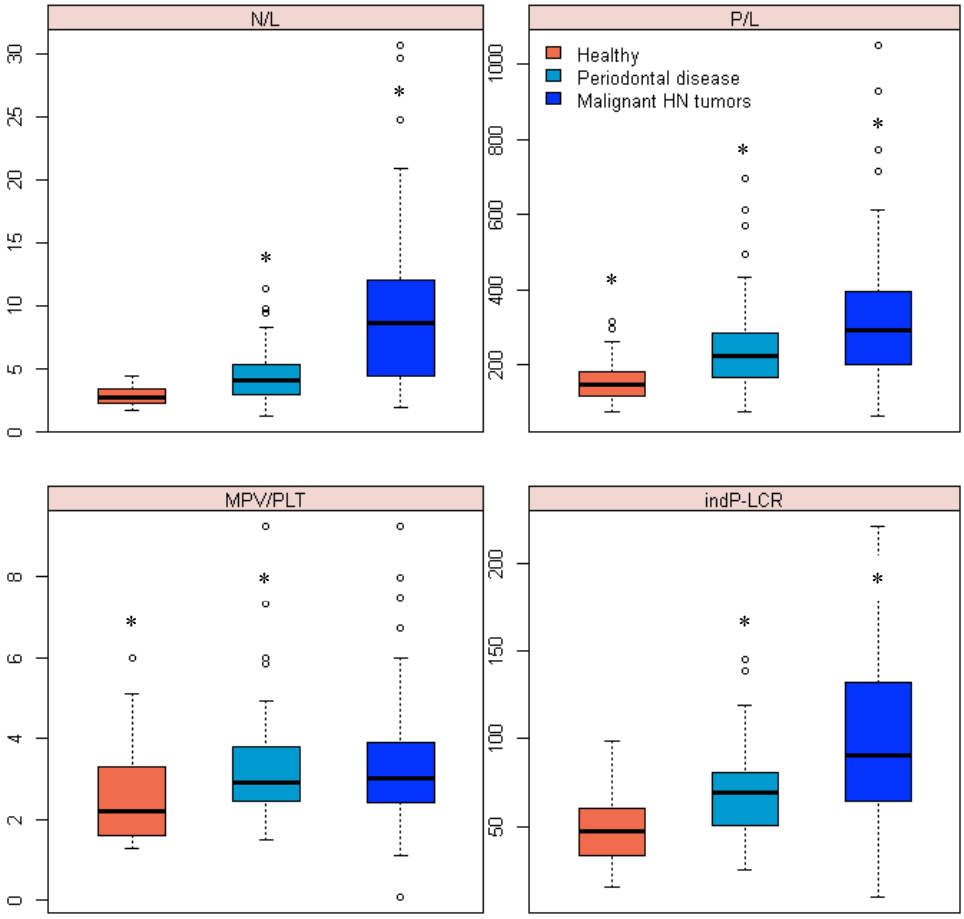


Figure 2: N/L, P/L, MPV/PLT ratios and PLCRi between groups of dogs investigated. N/L and PLCRi were significantly different between dogs with PD and NHNC and between healthy dogs and dogs with PD. P/L was significantly different between all groups of dogs investigated. MPV/PLT was significantly different between healthy dogs and dogs with PD and between healthy dogs and dogs with NHNC. * indicates significant difference.

Healthy dogs always exhibited the lowest values of CBC indices while dogs with NHNC were demonstrated with the highest values of parameters investigated. The values of N/L, P/L, MPV/PLT ratios and PLCRi of dogs with stage 3,4 PD (moderate to advanced periodontitis) were always between the values of healthy controls and the values of dogs with NHNC.

6.1.3 Univariate analysis of complete blood count indices (N/L, P/L and MPV/PLT ratios, PLCRi) and overall survival of dogs with different types of head and neck tumours

Table 4: *Univariate analysis of complete blood count indices (N/L, P/L and MPV/PLT ratios, PLCRi) and overall survival of dogs with different types of head and neck tumours*

Variable	*Carcinoma (n=31)*	*Melanoma (n=19)*	*Sarcoma (n=24)*	*Epulis (n=18)*	*P*
N/L	11 (1.86 - 24.75)	9.8 (3.8 - 30.7)	6.58 (3.27 - 17)	3.75 (2.28 - 14.2)	<0.001
P/L	298 (62.13 - 1051)	380 (100.6 - 591)	265 (120 - 610)	278.6 (73.7 - 771.7)	0.070
MPV/PLT	3.09 (1.13 - 9.26)	2.67 (2.06 - 7.5)	3.19 (1.61 - 7.98)	3.02 (0.08 - 4.47)	0.549
P-LCRi	100.9 (9.46 - 210.4)	125.5 (68.9 - 221.0)	65.0 (28.39 - 178.61)	77.08 (23.6 - 147.4)	<0.001
OS	232 (35 - 1143)	216 (31 - 635)	193.5 (81 - 1055)	912 (405 - 1668)	<0.001

The table 4 represents the results of univariate statistical analysis (Kruskal-Wallis test) revealing differences of CBC indices (N/L, P/L and MPV/PLT ratios and PLCRi) between groups of dogs with different types of head and neck tumours investigated.

No significant differences in P/L and MPV/PLT between groups of dogs with NHNC were detected but significant differences were confirmed for N/L ratio, PLCRi and overall survival (p=0.001). The lowest N/L ratios were demonstrated in dogs with oral benign lesions in comparison with dogs with malignant HNC. The N/L ratio of dogs with benign oral lesions was similar to the N/L ratio of dogs affected with stage 3,4 PD (moderate to advanced periodontitis) (Table 2 and Table 4). Univariate statistical analysis confirmed the differences in PLCRi between groups of dogs investigated. The lowest PLCRi values were detected in dogs with epulides and

sarcomas while the highest values were demonstrated in dogs with carcinomas and melanomas (Table 4).

According to the univariate and multivariate statistical analysis the overall survival of dogs with benign oral lesions was significantly longer in comparison with dogs with malignant HN conditions. Significant differences when comparing a group of dogs with benign lesions with a group of dogs with carcinomas or melanomas or sarcomas were detected ($p<0.001$).

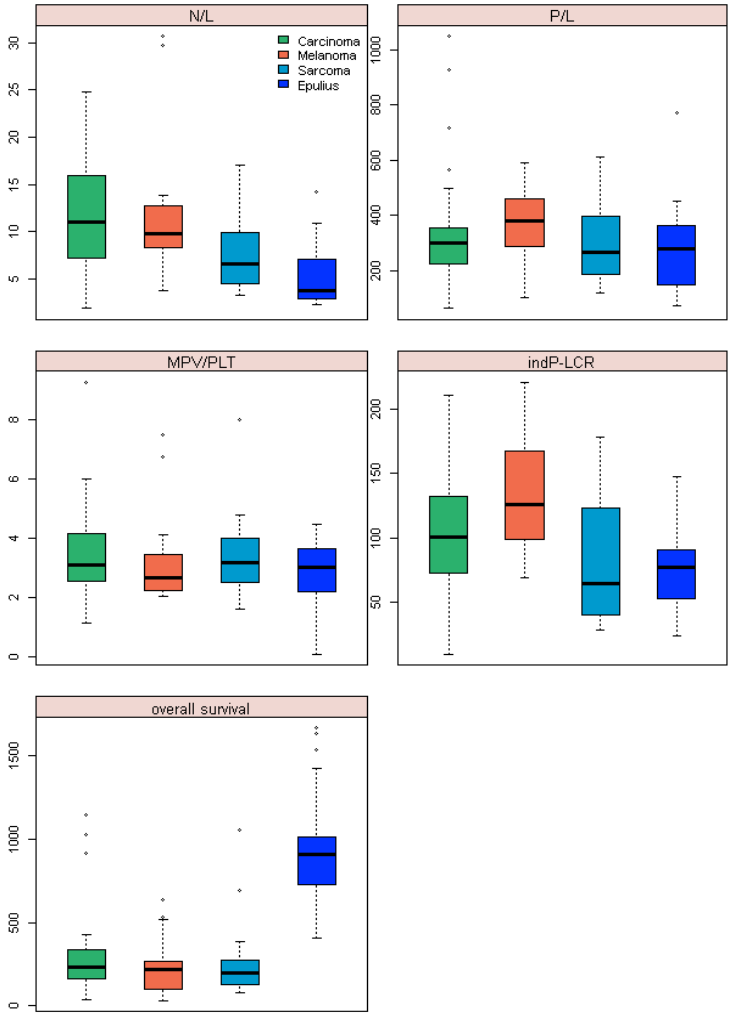


Figure 3: N/L, P/L and MPV/PLT ratios, PLCRi and overall survival comparing groups of dogs with different head and neck tumour types.

6.1.4 Multivariate analysis, investigating the impact of complete blood count indices (N/L, P/L and MPV/PLT ratios, PLCRi) on overall survival of dogs with neoplastic head and neck conditions

Table 5: *Multiple linear regression analysis investigating the impact of variables (N/L, P/L and MPV/PLT ratios, PLCRi) on overall survival in dogs with neoplastic head and neck conditions.*

Variable	*Coef.*	*SE (Coef.)*	*P*
N/L	-5.20	6.66	0.437
P/L	-0.05	0.24	0.847
MPV/PLT	4.26	25.68	0.869
P-LCRi	-0.75	0.74	0.311

The results of multiple linear regression analysis are revealing no significant impact of variables (N/L, P/L and MPV/PLT ratios and PLCRi) on the overall survival of patients investigated (Table 5). According to univariate and multivariate statistical analysis dogs affected with benign lesions had significantly longer OS survival in comparison with dogs affected with neoplastic head and neck conditions ($p<0.001$). Patients with benign lesions with significantly longer OS exhibited the lowest values of the CBC indices in comparison with dogs with NHNC where the values of CBC indices were demonstrated to be higher. There were no significant differences in OS between groups of dogs with different tumours types (carcinoma, melanoma, sarcoma) and this is ascribed to the comparable overall survival of all dogs (193-232 days) with these types of head and neck neoplasia. According to multiple linear regression statistical analysis the impact of N/L, P/L, MPV/PLT ratios and PLCRi on the overall survival of investigated group of dogs with neoplastic head and neck conditions was not confirmed.

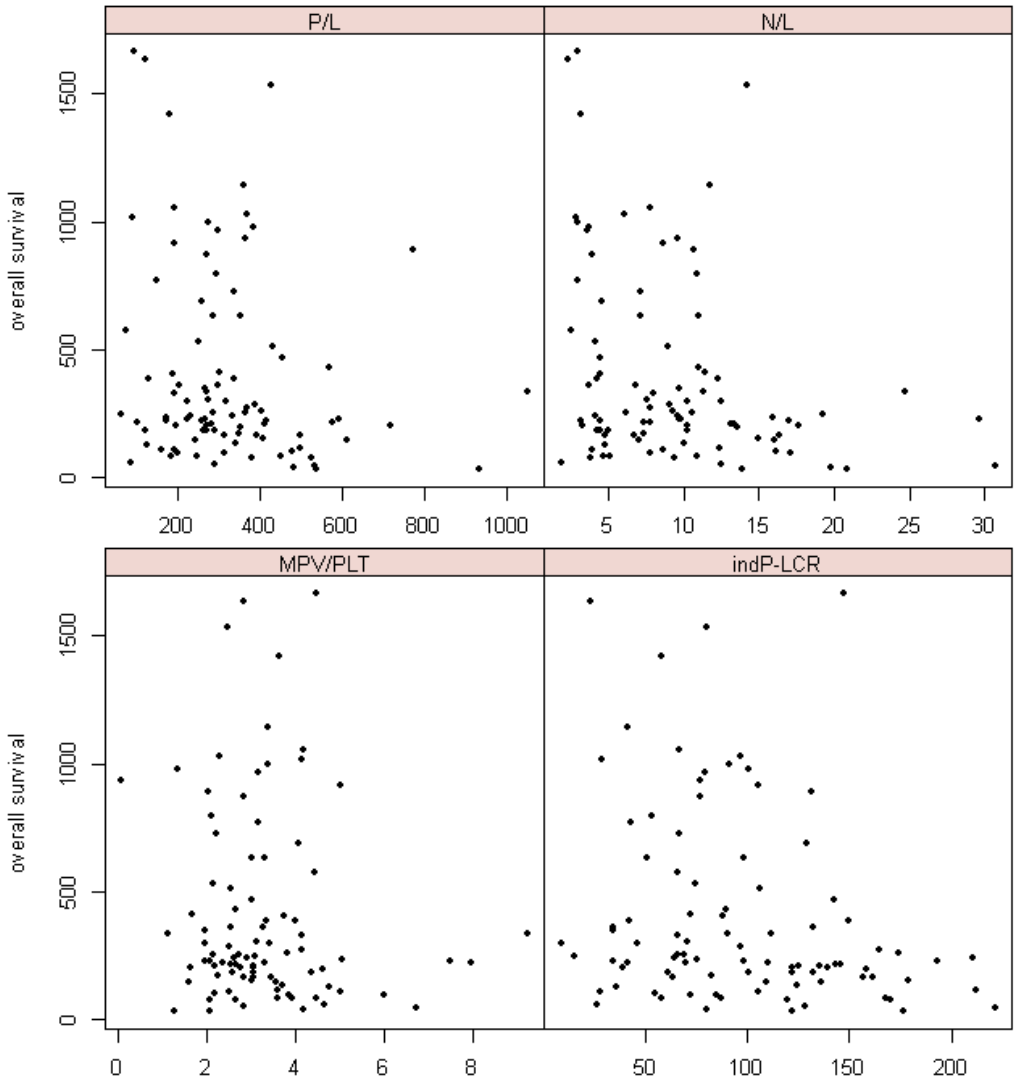


Figure 4: Relationship between overall survival and CBC indices (P/L ratio, N/L ratio, MPV/PLT ratio and PLCRi) of dogs with neoplastic head and neck conditions. x-axis indicates the values of CBC indices investigated. y-axis is presenting the overall survival time (days).

6.2 Systemic levels of Tregs and other lymphocyte T and B cell sets and subsets in dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis)

6.2.1 Patients

A group of dogs with stage 1 PD (gingivitis) consisted of 22 dogs. 17 dogs (77%) were mixed breed dogs and 5 (22%) were pure breed dogs. There were 12 males (7 neutered and 5 intact) and 10 females (5 neutered and 5 intact). The mean age of dogs with stage 1 PD (gingivitis) was 8.1±2.6 years (range 5-12 years) and the mean weight was 16.5±8.6 (range 6.1-29.4 kg). A group of dogs affected with stage 3,4 PD (moderate to advanced periodontitis) consisted of 27 dogs. There were 22 mixed breed dogs (81%) and 5 pure breed dogs (19%). There were 18 females (15 neutered and 3 intact) and 9 males (7 neutered and 2 intact). The mean age of patients with stage 3,4 PD (moderate to advanced periodontitis) was 10.2±2.4 years (range 7-12 years) and the mean weight was 7.6± 4.9 (range 5.1-17.4 kg) (Table 6). There was a significant difference between the average age and weight of the dogs ($p<0.05$). Dogs with stage 3,4 PD (moderate to advanced periodontitis) were older and weighed less in comparison with dogs with stage 1 PD (gingivitis). All dogs investigated were pet dogs. Dogs of both of the groups investigated were otherwise healthy and no other systemic diseases that could have an effect on the parameters investigated were detected.

Table 6: Characteristics of dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis)

Variables	*Dogs with stage 1 PD (n=22)*	*Dogs with stage 3 to 4 PD (n=27)*
Mixed breed	17 (77%)	22 (81%)
Pure breed	5 (22%)	5 (19%)
Male	12	9
Neutered	7	7
Intact	5	2
Female	10	18
Neutered	5	15
Intact	5	3
Mean age	8.1 +/- 2.6 years (5-12 years)	10.2+/-2.4 years (7-12 years)
Mean weight	16.5 +/- 8.6 kg (6.1-29.4 kg)	7.6+/-4.9 kg (5.1-17.4 kg)

6.2.2 Periodontal status

22 dogs were affected with stage 1 PD and 27 dogs with stage 3,4 PD (moderate to advanced periodontitis) with 25-50% or >50% of the teeth were affected. When comparing both groups of dogs investigated (dogs with stage 1 PD and dogs with stage 3,4 PD) there were evident changes in the values of clinical parameters of periodontitis. The values of plaque index (PI), bleeding on probing (BOP), gingival index (GI) and periodontal pocket depth (PPD) are presented in Table 7. Dogs with stage 3,4 PD (moderate to advanced periodontitis) were diagnosed with higher values of clinical parameters associated with periodontitis in comparison to dogs with stage 1 PD (gingivitis). There were significant differences in these values between the two groups. Dogs with stage 3,4 PD (moderate to advanced periodontitis) compared to dogs with stage 1 PD (gingivitis) had more plaque and calculus formation, more

pronounced inflammation of gingiva and bleeding on probing as well as deeper periodontal pockets. The values obtained for the group of dogs with stage 1 PD (gingivitis) confirmed a relatively healthy periodontium in 22.9% (5/22) of dogs where only localized gingivitis was detected. 77.1 % of dogs (17/22) had generalized gingivitis without clinical attachment loss (Table 7).

Based on the cytological examination of fine needle aspiration (FNA) of regional lymph nodes the cellular population consisted of small mature lymphocytes and lymphoblasts with low to moderate numbers of plasma cells, macrophages, monocytes and neutrophils in the majority of dogs with moderate to advanced periodontitis (stage 3,4 PD).

Table 7: Values of clinical parameters of periodontitis (PI, BOP GI and PPD) of dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis).

Variables	*Stage 1 PD (n=22)* *Mean ± SD*	*Stage 3,4 PD (n=27)* *Mean ± SD*	*P-value*
PI	0.32 ± 0.58	0.92 ± 0.48	< 0.001
BOP	0.20 ±0.41	0.52 ±0.53	< 0.001
GI	0.32 ± 0.40	0.72 ± 0.70	< 0.001
PPD (mm)	1.5 ± 0.71	5.2 ± 0.54	< 0.001

Mann-Whitney test; statistical significance at $p<0.005$; PD = periodontal disease; PI = plaque index; BOP = bleeding on probing; GI = gingival index; PPD = periodontal pocket depth (measured at 4 sites; distal, mesial, buccal and palatinal/lingual)

6.2.3 Results of flow cytometric analysis

Peripheral blood and regional lymph node samples were collected from dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis) and evaluated to determine the peripheral blood Tregs ($CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$) and other lymphocyte subpopulations ($CD4^+$, $CD8^+$ and $CD5^+$ T cell-subsets and $CD21^+$ B cells) expressed as the percentage of each cell type. Tregs were expressed as the percentage of $CD4^+$ expressing $CD25^+$ ($CD4^+CD25^+$) and $CD4^+CD25^+$ Tregs expressing $FOXP3^+$ cells ($CD4^+CD25^+FOXP3^+$) within the overall $CD4^+$ T cell population.

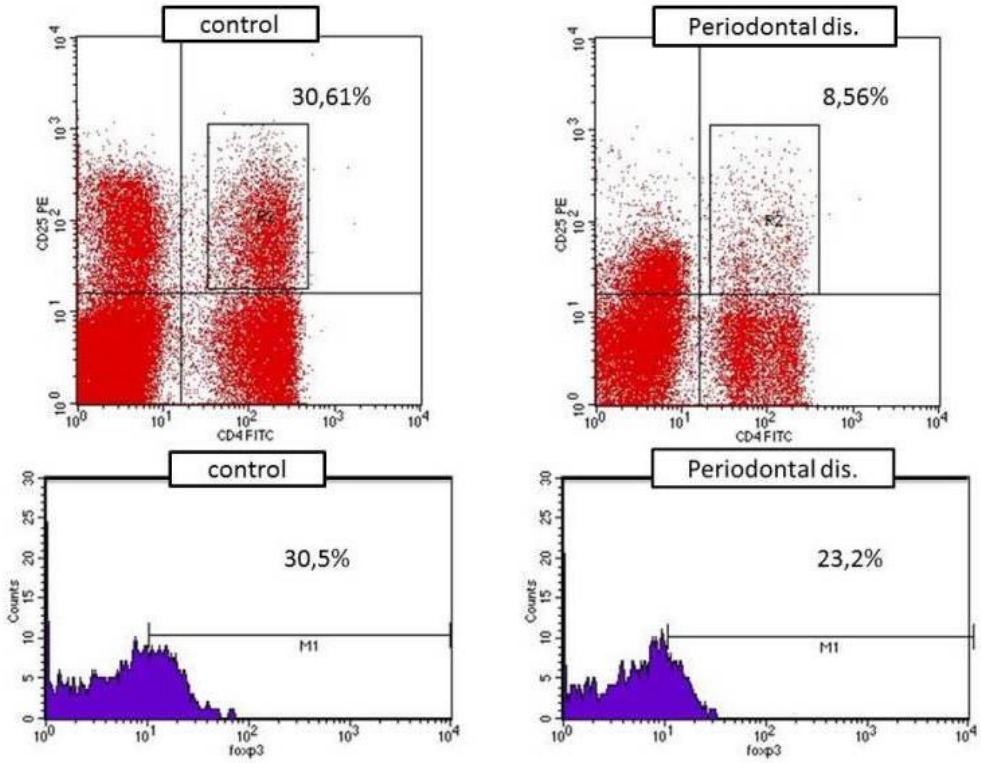


Figure 5: Peripheral blood mononuclear cells (PBMCs) were used in a two colour flow-cytometric analysis to identify surface expression of CD4, CD25 and intracellular expression of transcription factor FOXP3 in dogs with stage 1 PD (control) and dogs with stage 3,4 PD (moderate to advanced periodontitis). Lymphocytes of dogs with stage 1 PD and dogs with stage 3,4 PD were stained with anti-CD4 in the FITC channel (x-axis) and anti-CD25 in the PE channel (y-axis). Two-parameter (dual-colour fluorescence) histogram represents cells that were first gated on a forward/side scatter plot to specify the lymphocyte population for further analysis. Cells negative for both markers (CD4 and CD25) are on the bottom left, CD4 positive cells are on the bottom right and CD25 positive cells on the top left. Cells positive for both markers ($CD4^+CD25^+$ cells) are on the top right (R2) and those were used in subsequent analysis of $CD4^+CD25^+$ cells expressing FOXP3. A histogram for FOXP3 gated on the $CD4^+CD25^+$ population shows $CD4^+CD25^+$ cells expressing FOXP3. The intensity of the fluorescence increases from left to right (x-axis) and from bottom to top (y-axis). The two histograms present results of the FOXP3 expression in $CD4^+CD25^+$ Tregs individually. The x-axis represents the intensity of the fluorescence, the y-axis the number of cells. The bar stands (M1) for the amount of FOXP3 positive cells.

6.2.3.1 Peripheral blood regulatory T cells and other lymphocyte subpopulations investigated by flow cytometry in dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis)

Table 8: Complete blood count parameters (CBC) and peripheral blood regulatory T cells and other lymphocyte subpopulations in dogs with stage 1 PD (gingivitis) and canine patients with stage 3,4 PD (moderate to advanced periodontitis)

Variable	Stage 1 PD (n=22)		Stage 3,4 PD (n=27)		P-value
	Mean ± SD	Median	Mean ± SD	Median	
WBC ($x10^9$/L) (ref. 6-15)	13.0± 2.8	12.4 (8.6-18.4)	10.4±5.5	9.3 (3.7-26.2)	0.009
LYM (%) (ref. 13-30)	23.0±8.1	24.1 (11.9-36.2)	16.1±15.6	15.6 (7.7-26.7)	0.012
LYM ($x10^9$/L) (ref. 1-3.6)	2.9±1.1	2.7 (1.5-6.1)	1.7±0.6	1.7 (0.7-2.8)	<0.001
$CD5^+$ (%)	59.4±14.8	62.9 (30.7-83.1)	12.8±4.0	12.8 (4.2-20.8)	<0.001
$CD21^+$ (%)	22.9±13.9	21.4 (5.2-54.8)	4.4±2.6	4.6 (0.7-10.9)	<0.001
$CD4^+$ (%)	35.7±9.0	34.9 (18.6-55.0)	9.5±2.3	9.4 (6.1-14.1)	<0.001
$CD8^+$ (%)	20.0±9.3	19.0 (7.6-39.6)	4.4±1.8	4.3 (1.1-7.4)	<0.001
CD4/8	1.7±1.1	1.83 (0.8-31.4)	2.1±1.2	2.1 (1.2-5.9)	0.052
$CD4^+CD25^+$ (%)	16.9±7.1	14.89 (8.0-31.4)	5.2±2.4	5.3 (1.1-9.2)	<0.001
$CD4^+CD25^+FOXP3^+$ (%)	34.1±7.6	33.1 (20.3-51.7)	20.9±8.6	17.8 (9.8-34.7)	<0.001

Values represent mean ± SD and median values; Mann-Whitney test; statistical significance at $p<0.005$.

6.2.3.2 Regional lymph node regulatory T cells investigated by flow cytometry in dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis)

Table 9: Regional lymph node regulatory T cells investigated by flow cytometry in dogs with stage 1 PD (gingivitis) and canine patients with stage 3,4 PD (moderate to advanced periodontitis).

Variable (%)	Stage 1 PD (n=22)		Stage 3,4 PD (n=27)		P-value
	Mean ± SD	Median	Mean ± SD	Median	
$CD4^+$	39.24±8.1	34.9 (18.6-55.0)	44.6±12.68	51.56 (29.23-70.2)	0.023
$CD4^+CD25^+$	33.27±4.1	22.77 (7.0-34.4)	4.94±2.41	5.23 (3.43-6.8)	<0.001
$CD4^+CD25^+FOXP3^+$	41.42±5.4	49.13 (21.3-66.6)	13.45±9.25	11.58 (3.81-19.3)	<0.001

Values represent mean ± SD and median values; Mann-Whitney test; statistical significance at $p<0.005$.

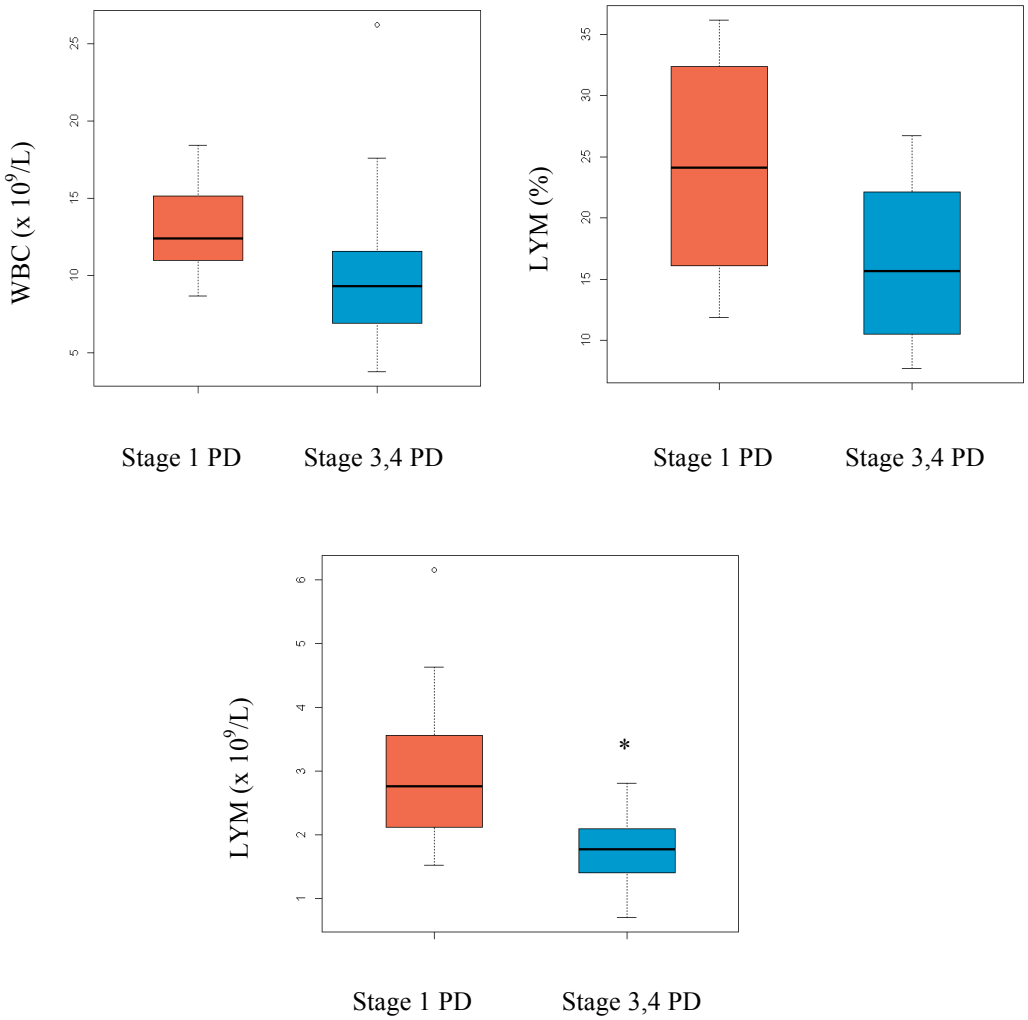


Figure 6: Absolute numbers of white blood cells (WBC), the relative and absolute number of lymphocytes of dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis). * indicates significant difference between groups of dogs investigated.

No statistically significant differences in the absolute number of white blood cells and the relative numbers of lymphocytes were detected between dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis) ($p=0.009$; $p=0.012$, respectively). Significant decrease of the absolute numbers of lymphocytes in dogs with stage 3,4 PD (moderate to advanced periodontitis) in comparison with dogs with stage 1 PD was detected ($p<0.001$) (Table 8; Figure 6).

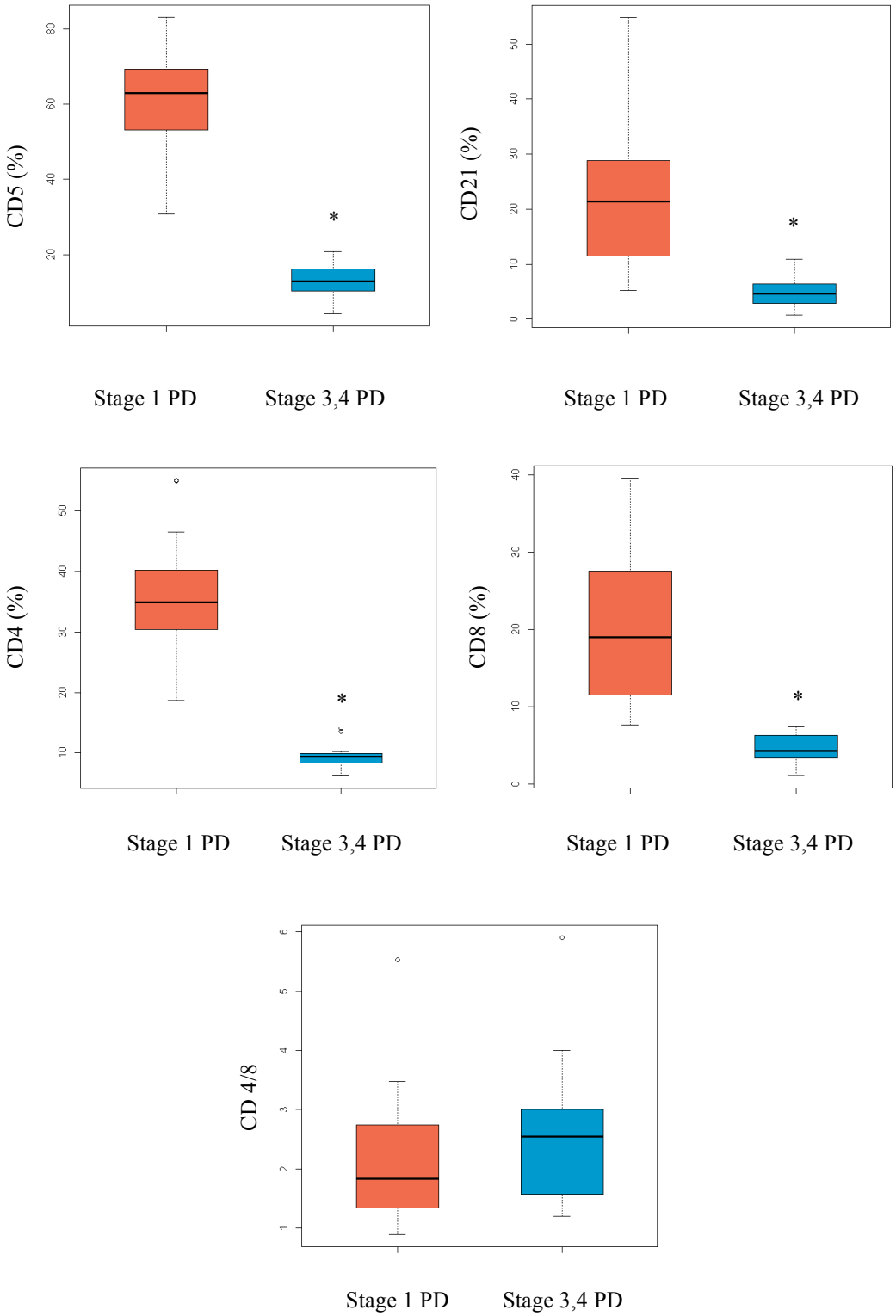


Figure 7: Percentage of peripheral blood $CD5^+$ T lymphocytes, $CD21^+$ B lymphocytes, $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells and CD4/8 ratio dogs with stage 1 PD (gingivitis) and dogs with stage 3,4 PD (moderate to advanced periodontitis). * indicates significant difference between groups of dogs investigated.

Significant differences in peripheral blood T and B cell sets and subsets were detected in dogs with stage 1 PD (gingivitis) and stage 3,4 PD (moderate to advanced periodontitis). The percentages of $CD5^+$, $CD4^+$, $CD8^+$ T-cell subsets and $CD21^+$ B cells were significantly decreased in dogs with stage 3,4 PD (moderate to advanced periodontitis) as compared to dogs with stage 1 PD (gingivitis). The mean percentages of $CD5^+$, $CD4^+$, $CD8^+$ T-cell subsets and $CD21^+$ B cells in dogs with stage 1 PD (gingivitis) were 59.42% (± 1.84), 35.72% (± 9.05), 20.01% (± 9.33) and 22.9% (± 13.97), respectively. The mean percentages of $CD5^+$, $CD4^+$, $CD8^+$ T-cell subsets and $CD21^+$ B cells in dogs with stage 3,4 PD (moderate to advanced periodontitis) were 12.89% (± 4.02), 9.56% (± 2.39), 4.48% (± 1.89), 4.40% (± 2.63), respectively. The mean ratio of CD4/8 in dogs with stage 3,4 PD (moderate to advanced periodontitis) was 2.14 (±1.25) versus 1.78 (±1.13) of dogs with stage 1 PD (gingivitis) and was not significantly altered (P=0.052) (Table 8; Figure 7).

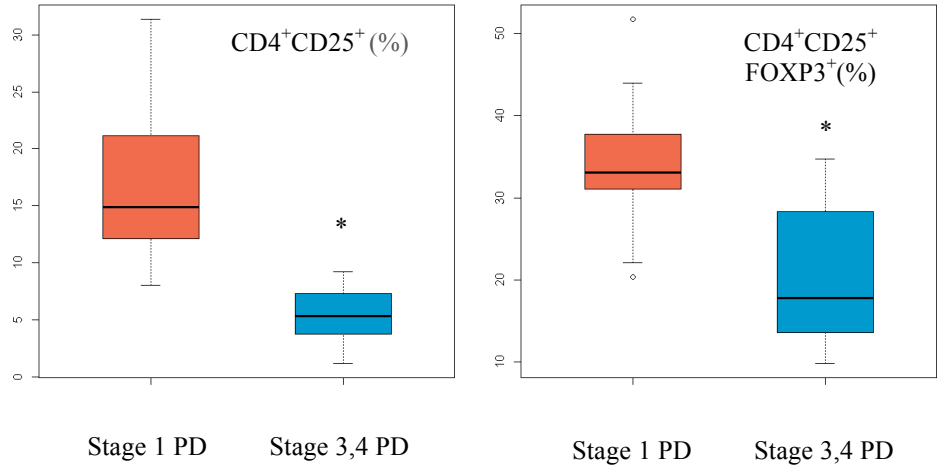


Figure 8: Percentage of peripheral blood regulatory T cells $CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$ dogs with stage 1 PD (gingivitis) and in dogs with stage 3,4 PD (moderate to advanced periodontitis). * indicates significant difference between groups of dogs investigated.

Significant differences in peripheral blood of Tregs were detected in dogs with stage 1 PD (gingivitis) and stage 3,4 PD (moderate to advanced periodontitis). Dogs with stage 3,4 PD (moderate to advanced periodontitis) were diagnosed with significantly lower percentages of Tregs ($CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$) in the blood as compared to dogs with stage 1 PD (gingivitis) ($p<0.001$). The mean percentage of total $CD4^+CD25^+$ (including $CD4^+$ T cells expressing low-medium-high CD25) in the blood of dogs with stage 3,4 PD (moderate to advanced periodontitis) was 5.24% (± 2.45) versus 16.97% (± 7.12) in the blood of control dogs ($p<0.001$). The mean percentage of $CD4^+CD25^+$ expressing $FOXP3^+$ in the blood of dogs with stage 3,4 PD (moderate to advanced periodontitis) was 20.97% (± 8.65) versus 34.18% (± 7.62) in the blood of dogs with stage 1 PD (gingivitis) ($P<0.001$) (Table 8). Of the total population of $CD4^+CD25^+$ T cells of dogs with stage 1 PD (gingivitis), approximately 1/3 (33.11%) were $CD4^+CD25^+$ T cells expressing FOXP3. $CD4^+CD25^+$ T cells expressing FOXP3 represented 4.96% of all $CD4^+CD25^+$ T cells and 1.75 % of all $CD4^+$ T cell population in peripheral blood (Table 8; Figure 8).

Dogs with stage 3,4 PD (moderate to advanced periodontitis) were diagnosed with significantly lower percentages of regional lymph node Tregs in comparison with dogs with stage 1 PD ($p<0.001$). The mean percentage of total $CD4^+CD25^+$ in the regional lymph nodes of dogs with stage 3,4 PD (moderate to advanced periodontitis) was 4.94% (± 2.41) versus 33.27% (± 4.13) in the lymph nodes of dogs with stage 1 PD (gingivitis). The mean percentage of $CD4^+CD25^+FOXP3^+$ in the regional lymph nodes of dogs with stage 3,4 PD (moderate to advanced periodontitis) was 13.45% (± 9.25) versus 41.42% (± 5.47) in the regional lymph nodes of dogs with stage 1 PD (gingivitis) (Table 9). The values of Tregs in regional lymph node in comparison with peripheral blood $CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$ Tregs of dogs with stage 1 PD (gingivitis) were slightly higher. The same was true when comparing the values of regional lymph node Tregs with peripheral blood Tregs of dogs with stage 3,4 PD (moderate to advanced periodontitis) where similar reduction of $CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$ Tregs were demonstrated.

There was a high positive correlation between regulatory T cells ($CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$ Tregs) in dogs with stage 3,4 PD (moderate to advanced periodontitis) (r=0.71; p=0.006). The correlations between $CD4^+$, $CD8^+$, $CD21^+$, $CD5^+$ and $CD4^+CD25^+$ (r=0.94, r=0.91; r=0.97; r=0.81, respectively) were interpreted as very high. The differences were statistically significant ($p<0.00001$). Less correlations, interpreted as moderate to high, were detected between $CD4^+$, $CD8^+$, $CD21^+$, $CD5^+$ and $CD4^+CD25^+FOXP3$ (r=0.52, p=0.002; r=0.38, $p<0.00001$; r=0.45, p=0.008; r=0.27, p=0.12, respectively).

6.3 Immunohistochemical expression of Ki-67 and VEGFR-2 in canine inflammatory, benign and malignant head and neck conditions

6.3.1 Epidemiology and clinical history

Fifty-four cases of canine inflammatory, benign and malignant head and neck conditions (the majority were surgical samples) met the inclusion criteria. Clinical records were complete in all cases as all the patients with inflammatory, benign and malignant head and neck conditions were treated at Animal Hospital Postojna, Slovenia. Follow-up was available for all of them. There were 25 males (46%) and 29 (54%) females and the median average age was 12.7 years (range, 7 to 15 years). 9 males were castrated and 14 females were spayed. There were 26 mixed breed and 28 pure breed dogs.

Dogs with inflammatory and benign oral conditions represented 37% of cases (20/54) while dogs with malignant head and neck conditions represented 63% of cases (34/54). Benign cases consisted of epulis (n=11) and stomatitis (n=9) cases, representing 21% and 17% of all cases, respectively. Malignant head and neck cases consisted of 17 epithelial neoplasia (7 SNSCC, 10 oral SCC) and 7 malignant melanoma and 10 oral sarcoma cases. Oral sarcomas consisted of 6 oral osteosarcomas, 1 oral chondrosarcoma, 1 oral mixosarcoma, 1 oral rhabdomyosarcoma and 1 oral anaplastic sarcoma. Of 9 oral SCC there were 4 affecting the maxilla, 3 the mandible and 2 the tongue. All malignant melanomas were arising from the oral cavity affecting the mandible (3) or maxilla (3) with one affecting the buccal mucosa. Osteosarcomas were affecting the mandible (n=6) and maxilla (n=4) (Table 10).

Regional lymph nodes metastases were present in 6 of 34 (18%) malignant cases (in 4 dogs with MM, 1 dog with rhabdomyosarcoma and 1 dog with oral SCC). Distant metastases were presented in 4 of 34 (12%) malignant cases, that is in 3 dogs with oral MM and in 1 dog with oral rhabdomyosarcoma. All had pulmonary metastases.

At clinical presentation 5 of 34 (15%) dogs had stage I, 12 (35%) dogs stage II, 13 dogs (38%) stage III and 4 (12%) dogs stage IV. More specifically, among 17 dogs with epithelial tumours 3 (18%) had stage I, 6 (35%) stage II, 5 (29%) stage III and 3 (18%) stage IV. Among dogs with MM and sarcomas, 2 of 17 dogs (12%) had stage I, 6 (35%) stage II, 8 (47%) stage III and 1 (1%) stage IV.

Table 10: Distribution of dogs according to the type of inflammatory, benign and malignant head and neck conditions.

Inflammatory, benign and malignant HN conditions in dogs	**N=54**
Inflammatory and benign HN conditions	20 (37%)
Benign (epulides)	**11(21%)**
Inflammatory (stomatitis)	**9 (17%)**
Malignant HN conditions	34 (63%)
Epithelial (SCC)	17
Oral	**10 (18%)**
SN (sinonasal)	**7 (13%)**
MM + sarcoma	17
MM (malignant melanoma)	**7 (13%)**
SAR (sarcoma)	**10 (18%)**

6.3.2 Histopathological characteristics of inflammatory, benign and malignant head and neck conditions in dogs

Epulides

The neoplasms were composed of loosely arranged streams of spindle to stellate cells separated by abundant collagen matrix within a well-vascularized stroma. Neoplastic cells had indistinct borders with scant to moderate amounts of eosinophilic fibrillary cytoplasm. Nuclei were irregularly oval to elongate with moderately stippled chromatin and an indistinct nucleolus. The mitotic rate was less than 1/10 HPF. There were rare small subepithelial and perivascular accumulations of plasma cells, fewer lymphocytes and occasional neutrophils. No necrosis was present.

Stomatitis

Superficial portion of mucosa was characterized by moderate epithelial hyperplasia associated with mild to moderate chronic infiltrates and lymphocytic exocytosis on the epithelial basal layer. The underlying stroma was composed of fibrous tissue with mild perivascular lymphoplasmacytic infiltrate.

Sinonasal/oral SCC

The samples were composed of atypical proliferation of neoplastic epithelial cell population arranged in cords and nests supported by a moderate fibro-vascular stroma**.** Neoplastic cells were polygonal, with poorly or moderately defined borders with moderate to abundant eosinophilic cytoplasm. Nuclei were round with marginal chromatin and a central round evident nucleolus. Anisocytosis and anisokaryosis were moderate and mitoses ranged from 0-24 per 10/HPF. Neoplastic cells showed diffuse squamous differentiation with accumulation of keratin. In the stroma and

associated keratin a mixed inflammatory population, composed mainly from neutrophils and occasional lymphocytes and plasma cells was present.

Osteosarcoma

The samples were composed of polygonal to spindle cells arranged in short interlacking streams and bundles often surrounding foci of eosinophilic homogenous to fibrillary matrix (tumour osteoid). Neoplastic cells had poorly demarcated cell borders and scant cytoplasm. Nuclei were ovoid and contained ovoid nuclei with stippled chromatin and 1-2 prominent nucleoli. Anisocytosis and anisokaryosis were present. Mitoses ranged from 2-18 per 10/HPF. Scattered throughout the neoplasm there were necrotic areas and few multicentre giant cells (osteoclasts).

Malignant melanoma

Atypical cellular population organized in nests and irregular bundles was observed. The cells, with occasional poorly defined cell borders by moderate to abundant cytoplasm with occasional melanin granules were characterized. The nuclei were round to ovoid with a prominent nucleolus. The cells were highly pleomorphic with moderate to abundant eosinophilic cytoplasm and a round central nucleus with a prominent nucleolus. Some cells were multinucleated as well. Ansiocytosis and anisokaryosis were moderate to severe. The number of mitoses ranged from 0-24 per 10/HPF. There was also a mild to moderate amount of eosinophilic fibrillary matrix and multifocal areas of oedema and inflammation (eosinophils, lymphocytes, macrophages and occasional plasma cells).

Presence of necrosis

62% (21/34) of malignant head and neck tumours were negative for the presence of necrosis. 29% (5/17) of epithelial tumours and 47% (8/17) of MM and sarcoma cases

revealed the presence of necrosis. No significant differences in the presence of necrosis between head and neck carcinomas and MM and sarcoma cases were detected.

The number of mitosis

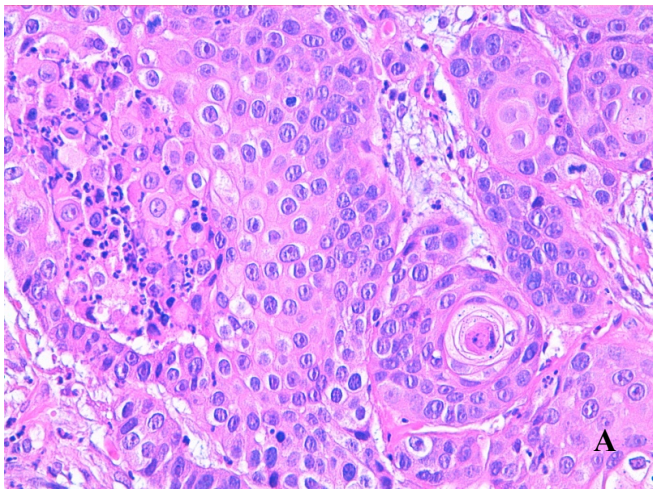
The number of mitoses ranged from 1 to 24 per 10/HPF. The mean number of mitoses in the majority of tumours was 1-6 per 10/HPF. No significant differences in the number of mitoses between head and neck carcinomas and MM and sarcoma cases were detected.

Tumour grade

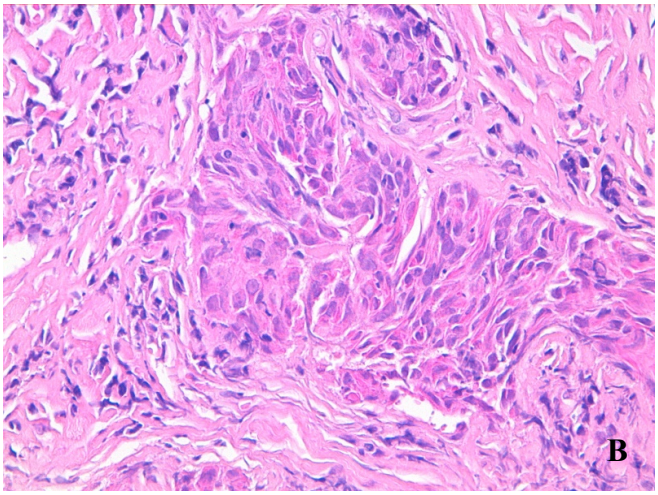
53% of epithelial tumours were grade I (9/17) and 47% (8/17) were grade II. No grade III was detected. Among MM and SAR cases, 59% (10/17) were low grade. There were 6 low grade SAR and 4 low grade MM. There were 7 high grade tumours (grade III tumours) consisting of 3 MM and 4 SAR (Table 11).

Table 11: Distribution of malignant head and neck tumours according to grade.

Variable	Epithelial tumours (n=17/34)	MM (n=7/34)	Sarcoma (n=10/34)
Grade I (low)	9 (26%)	4 (12%)	6 (18%)
Grade II (intermediate)	8 (23%)	0 (0%)	0 (0%)
Grade III (high)	0 (0%)	3 (9%)	4 (12%)



Grade I (40x, H&E)



Grade II (40x, H&E)

Figure 9: The difference between grade I (panel A) and grade II (panel B) of the head and neck squamous cell carcinoma on H&E stained tissue samples.

Panel A: Numerous cells with defined borders, eosinophilic cytoplasm with centrally placed nuclei are present. Nuclei are round, central, with finely stippled chromatin and a central round basophilic nucleolus. Anisocytosis and anisokaryiosis are moderate. Cells in the centre of neoplastic aggregates are in squamous differentiation. Single necrotic or acantholytic neoplastic cells are occasionally evident. Panel B: Neoplastic epithelial cells are arranged in cords and nests surrounded by a moderate fibrovascular stroma. Neoplastic cells are polygonal to elongated, with poorly defined borders and moderate to abundant eosinophilic cytoplasm. Nuclei are round to oval with marginated chromatin and a central round nucleolus. Anisocytosis and anisokaryosis are moderate. Diffuse squamous differentiation is evident with minimal accumulation of keratin.

6.3.3 Expression of Ki-67 and VEGFR-2

Table 12: Expression of Ki-67 and VEGFR-2 in 54 dogs with inflammatory, benign and malignant head and neck conditions.

Benign and malignant HN conditions in dogs	N (54)	Ki-67 (%) Median (range)	Ki-67 (%) Mean ± SD	Int.	VEGFR (%) Median (range)	VEGFR (%) Mean ± SD)	Int.
Benign (epulides) + inflammatory (stomatitis)	20	0.5 (0 - 15.7)	2.65 ± 4.1	Neg /+	Neg.	Neg.	Neg.
Benign (epulides)	11	0.1 (0 - 0.6)	0.16 ± 0.2	Neg/+	Neg.	Neg.	Neg.
Inflammatory (stomatitis)	9	4.3 (1 - 15.7)	5.68 ±4.58	Neg/+	Neg.	Neg.	Neg.
Malignant	34	14.2 (0 - 73.5)	19 ± 18.9	++/+++	1 (0 - 90)	13.9 ± 23.4	++/+++
Epithelial (SCC)	17	16.8 (0.1 - 73.5)	26.25 ± 23.8	++/+++	1 (0 - 81.5)	17.7 ± 24.5	+/++
Oral SCC	10	11.8 (0.1 - 38.0)	13.5 ±12.5	++	0 (0 - 1)	0.2 ± 0.42	Neg/+
SNSCC	7	55.4 (10.2 - 73.5)	44.47 ± 24.8	++/+++	42 (25 - 81.5)	42.8 ± 18.9	+++
MM + SAR	17	13.8 (0 - 29.1)	13.74 ± 9.08	++	1 (0 - 90)	10.0 ± 22.3	Neg/+
MM	7	20.9 (0 - 29.1)	16.7 ± 10.53	++	1 (1 - 30)	9.8 ± 11.89	+/++
SAR	10	10.9 (2.2 - 28.9)	11.62 ± 7.8	++	0 (0 - 90)	10.2 ± 28.2	Neg/+

Values represent median and mean values and ranges of the percentage of the expression of Ki-67 and VEGFR-2. SCC; squamous cell carcinoma; SNSCC: sino-nasal squamous cell carcinoma; MM: malignant melanoma; SAR: sarcoma; + (weak); ++ moderate; +++ (strong).

Table 13: Immunohistochemical (IHC) score obtained by distribution and labelling intensity of Ki-67 and VEGFR-2 in 54 dogs with inflammatory, benign and malignant head and neck conditions

Benign and malignant HN conditions in dogs	N (54)	Ki-67 IHC score Mean ± SD	Ki-67 IHC score Median (range)	VEGFR-2 IHC score Mean ± SD	VEGFR-2 IHC score Median (range)
Benign (epulides) + inflammatory (stomatitis)	20	0.4 ± 0.82	0 (0-2)	0 ± 0	0 (0)
Benign (epulides)	11	0.00 ± 0.00	0 (0)	0 ± 0	0 (0)
Inflammatory (stomatitis)	9	0.89 ± 1.5	0 (0-2)	0 ± 0	0 (0)
Malignant	34	2.82 ± 2.50	0 (0-2)	1.76 ± 2.3	1 (0-4)
Epithelial (SCC)	17	3.41 ± 3.28	2 (0-9)	1.89 ± 2.57	1 (0-8)
Oral SCC	10	1.80 ± 3.01	2 (0-4)	0.2 ± 3.21	0 (0-1)
SN SCC	7	5.70 ± 1.73	4 (1-8)	4.2 ± 1.45	4 (2-6)
MM	7	2.57 ± 1.51	2 (0-4)	1.43 ± 1.27	1 (0-4)
SAR	10	2.00 ± 0.94	2 (0-4)	1.8 ± 2.53	1 (0-8)

Values represent median and mean values and ranges of Ki-67 and VEGFR-2 IHC scores. SCC; squamous cell carcinoma; SNSCC: sino-nasal squamous cell carcinoma; MM: malignant melanoma; SAR: sarcoma.

Table 14: Differences in the expression of the percentage of Ki-67 and VEGFR-2 in 54 dogs with inflammatory, benign and malignant head and neck conditions.

Inflammatory, benign, malignant head and neck conditions	P-value	
	Ki-67 (%)	VEGFR-2 (%)
Benign (epulides) + inflammatory lesions (stomatitis) / malignant tumours	<0.001	<0.001
Benign (epulides) / inflammatory lesions (stomatitis)	<0.001	/
epithelial/MM+SAR	0.245	0.801
MM/SAR	0.760	0.461
Oral SCC/SNSCC	0.019	<0.001

Table 15: Differences in the IHC score of Ki-67 and VEGFR-2 in 54 dogs with inflammatory, benign and malignant head and neck conditions.

Inflammatory, benign, malignant head and neck conditions	P-value	
	Ki-67 IHC score	VEGFR-2 IHC score
Benign (epulides) + inflammatory lesions (stomatitis)/malignant tumours	<0.001	<0.001
Inflammatory (stomatitis) / malignant	0.009	0.002
Benign (epulides)/inflammatory (stomatitis)	0.019	/
Epithelial/MM	0.895	0.742
MM/SAR	0.327	0.839
Oral SCC/SN SCC	0.027	<0.001

6.3.3.1 Expression of Ki-67

The percentage of expression of Ki-67 was statistically significantly lower in benign HN lesions consisting of stomatitis and epulis samples in comparison with malignant head and neck conditions ($p<0.001$) (Figure 10).

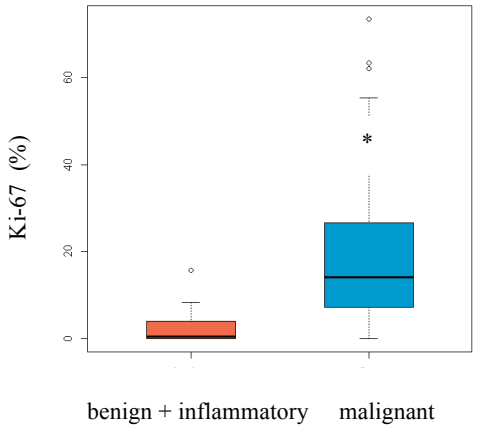


Figure 10: The percentage of expression of Ki-67 in dogs with benign and inflammatory HN lesions in comparison with malignant head and neck tumours in dogs; Mann-Whitney-Wilcoxon test shows significant difference* ($p<0.001$).

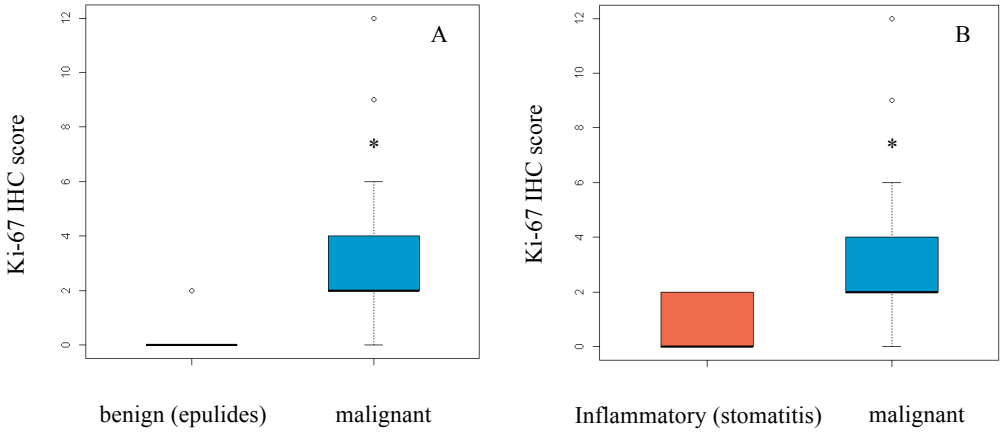


Figure 11: The Ki-67 IHC score in dogs with benign (epulides) (A) and inflammatory (stomatitis) HN lesions (B) in comparison with malignant head and neck tumours in dogs Mann-Whitney-Wilcoxon test shows significant difference* ($p<0.001$ and $p=0.008$, respectively).

The Ki-67 IHC score in dogs with benign (epulides) and inflammatory (stomatitis) HN lesions in comparison with malignant head and neck tumours was significantly lower ($p<0.001$ and $p=0.008$, respectively) (Figure 11 A, B). Samples of malignant head and neck conditions with positive Ki-67 labelling exhibited moderate-strong nuclear labelling while benign and inflammatory head and neck conditions exhibited negative or mild labelling. The higher percentage of the expression of Ki-67 increased with the intensity of labelling with the labelling more diffuse including cells in the centre of nests. The median score for the percentage of expression of Ki-67 for benign + inflammatory head and neck conditions was 0.5 % (95% CI 0-15.7%) and for malignant head and neck conditions 14.2 % (95% CI 0-73.5%). More specifically the median score for the percentage of expression of Ki-67 for epulides was 0.1 % (95% CI 0-0.6%) and stomatitis lesions 4.3 % (95% CI 1-15.7%). In tumours with low Ki-67 labelling the positive cells were located mainly in the periphery of the tumour cell nests while in tumours with high Ki-67 increasingly more cells towards the centre of the tumour nests exhibited positive labelling. Epulides expressed significantly lower percentage of Ki-67 and had significantly lower Ki-67 IHC score in comparison with inflammatory lesions (stomatitis) (Figure 12 A, B). The intensity for Ki-67 of both oral inflammatory and benign oral conditions was negative or mild.

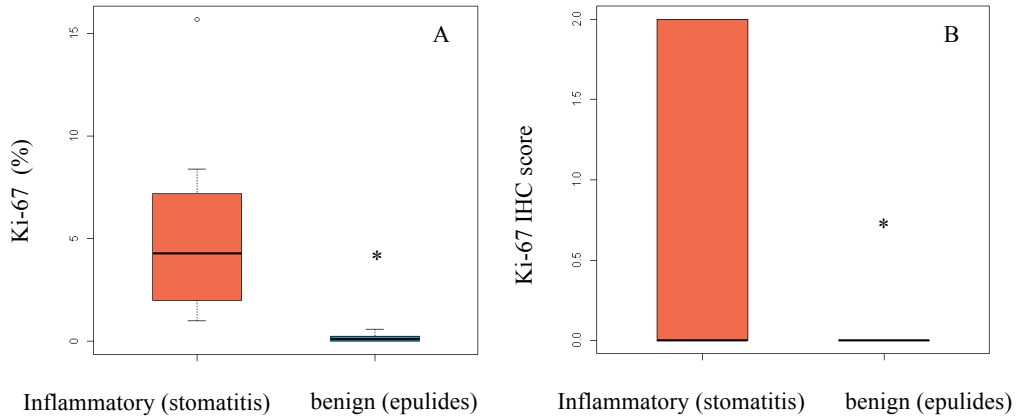


Figure 12: The expression of the percentage of Ki-67 (A) and Ki-67 IHC score (B) in dogs with inflammatory (stomatitis) compared to benign (epulides) head and neck lesions; Mann-Whitney-Wilcoxon test shows significant difference ($p<0.001$ and $p=0.018$, respectively).

Malignant HN tumours were expressing similar percentages of Ki-67. The difference in the expression of Ki-67 between epithelial HN tumours and a group of HN tumours consisting of MM and SAR was not significant (p=0.245) (Figure 13). No significant differences were detected when comparing the percentage of Ki-67 in epithelial *vs* MM (p=0.354) and MM *vs* SAR tumours (p=0.760). Similar results were obtained when comparing the Ki-67 IHC score between epithelial *vs* MM and MM *vs* SAR tumours (Figure 14 A, B).

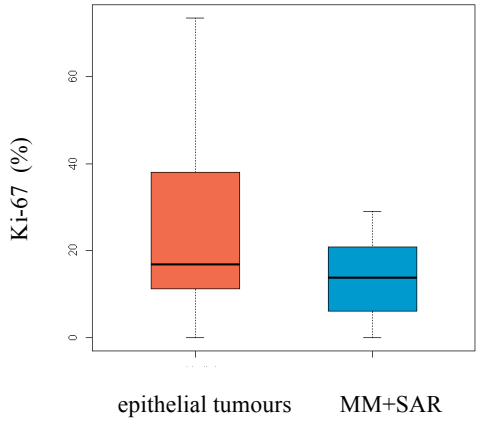


Figure 13: The expression of Ki-67 in dogs with epithelial and MM+SAR head and neck tumours; Mann-Whitney-Wilcoxon test shows no significant difference (p=0.245).

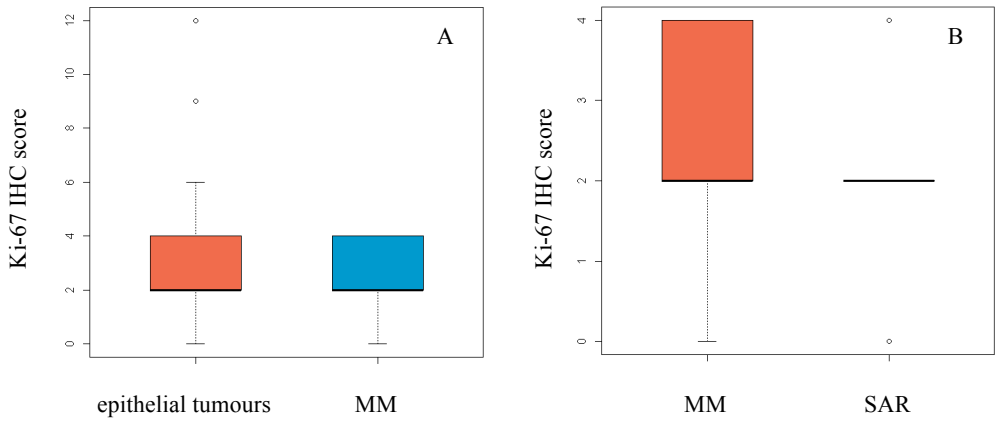


Figure 14: The Ki-67 IHC score in dogs with epithelial in comparison with MM tumours (A) and in dogs with MM and SAR (B); Mann-Whitney-Wilcoxon test shows no significant difference (p=0.895 and p=0.372, respectively).

When epithelial tumours were divided according to the location, the percentage of expression of Ki-67 was more pronounced in sino-nasal SCC tumours in comparison with SCC of the oral cavity and the difference was statistically significant (p=0.019) (Figure 15 A). The same was true for the Ki-67 IHC score of these tumour types (Figure 15 B).

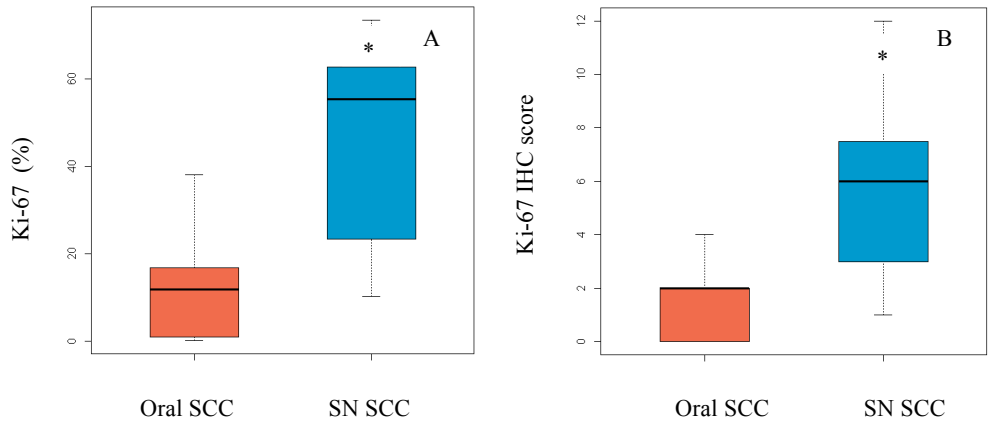


Figure 15: The expression of the percentage of Ki-67 (A) and Ki-67 IHC score (B) in dogs with oral SCC and sinonasal SCC; Mann-Whitney-Wilcoxon test shows significant difference* (p=0.019 and p=0.027, respectively).

Moderate or strong intensity of Ki-67 expression was present in all malignant head and neck tumours. There was a difference in the intensity of the expression between epithelial and mesenchymal head and neck tumours with epithelial sino-nasal SCC revealing strong pattern of the Ki-67 intensity while malignant melanoma and sarcoma tumours and epithelial oral SCC showed in the majority of cases moderate intensity pattern for Ki-67.

6.3.3.2 Expression of VEGFR-2

The expression of VEGFR-2 was not detected in inflammatory (stomatitis) and benign head and neck tumours (epulides) of dogs (Figure 16 A) while malignant head and neck tumours of dogs expressed VEGFR-2 (Figure 16 B). The difference in the expression of VEGFR-2 between dogs with benign head and neck tumours (epulides) + inflammatory head and neck conditions (stomatitis) compared with malignant head and neck conditions was significant ($p<0.001$) (Figure 16 B). The significant difference in VEGFR-2 IHC score when comparing benign head and neck tumours (epulides) or inflammatory head and neck conditions (stomatitis) with malignant head and neck conditions was detected as well ($p<0.001$ and $p=0.002$, respectively) (Figure 17 A, B).

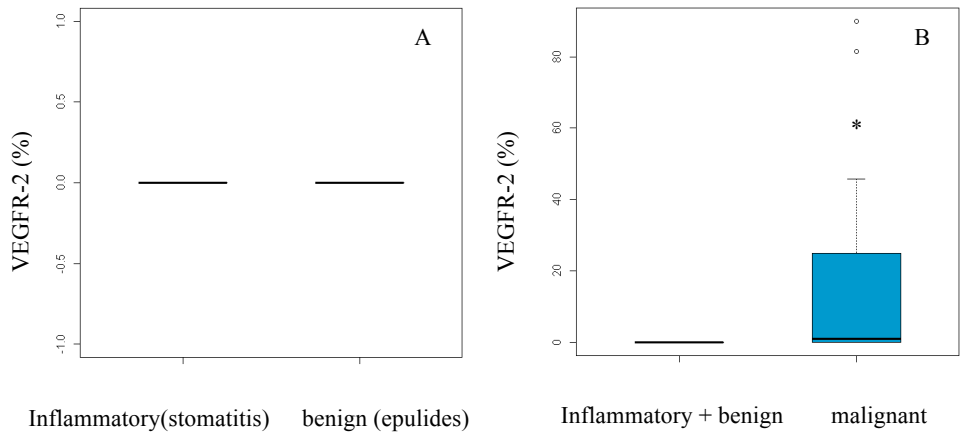


Figure 16: The expression of VEGFR-2 in dogs with inflammatory (stomatitis) + benign (epulis) head and neck conditions (A); Mann-Whitney-Wilcoxon test shows no difference. The % of VEGFR-2 in dogs with benign (epulides) + inflammatory (stomatitis) and malignant head and neck conditions (B); Mann-Whitney-Wilcoxon test shows significant difference* ($p<0.001$).

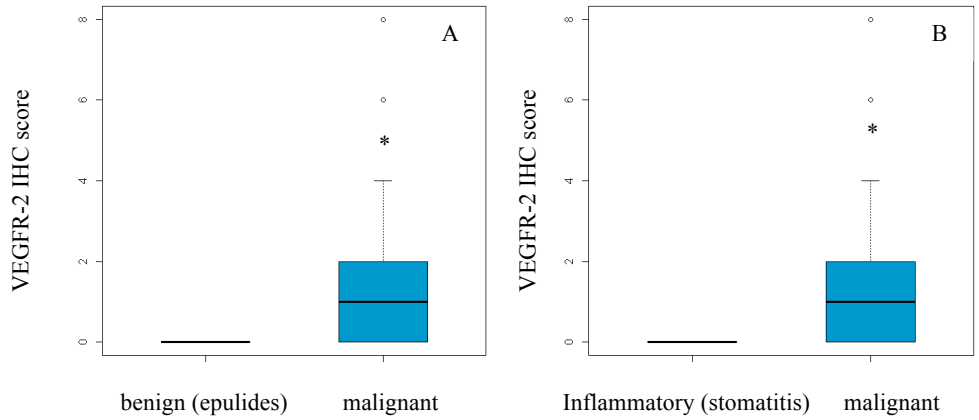


Figure 17: VEGFR-2 IHC score in dogs with benign (epulides) (A) or inflammatory (stomatitis) (B) compared to malignant head and neck conditions; Mann-Whitney-Wilcoxon test shows significant difference* ($p<0.001$ and $p=0.002$, respectively).

Malignant head and neck tumours exhibited cytoplasmatic and only occasionally membranous labelling for VEGFR-2. Positive immunoreactivity for VEGFR-2 was noted in the cytoplasm of tumour cells, which was the predominant location of the expression in the majority of cases. No nuclear labelling was seen. The labelling varied markedly in intensity as well as in area covered. The median score for VEGFR-2 malignant head and neck conditions was 1 % (95% CI 0-90.0%). Samples of malignant head and neck conditions with positive VEGFR-2 expression exhibited mild or moderate or strong labelling while labelling of inflammatory (stomatitis) and benign (epulides) head and neck conditions for VEGFR-2 was negative. The higher percentage of the expression of VEGFR-2 in malignant head and neck conditions increased with the intensity of labelling with the labelling more diffuse including cells in the centre of tumour nests.

The majority of malignant head and neck tumours (59%) (20/34 cases) stained positive for VEGFR-2. The expression of VEGFR-2 was widespread and differed regarding the histopathological type and location of malignant head and neck tumours. As epithelial and MM+SAR head and neck conditions expressed similar

percentage of VEGFR-2 the difference was not significant (p=0.801) (Figure 18). The intensity of expression was stronger in epithelial head and neck tumours when compared with MM+SAR cases. No significant difference in VEGFR-2 IHC score when comparing epithelial HN tumours with MM or SAR or MM with SAR was detected (p=0.741 and p=0.839, respectively) (Figure 19 A, B).

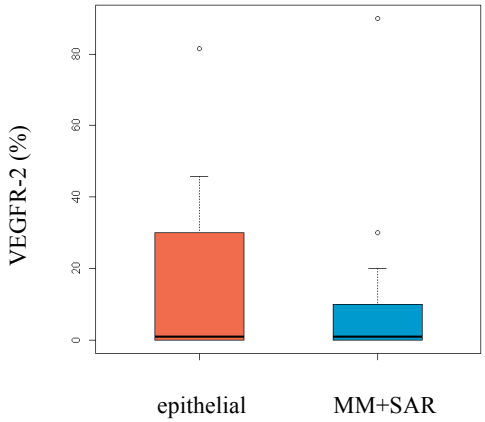


Figure 18: The expression of VEGFR-2 in dogs with epithelial and MM+SAR head and neck tumours; Mann-Whitney-Wilcoxon test shows no significant difference (p=0.801).

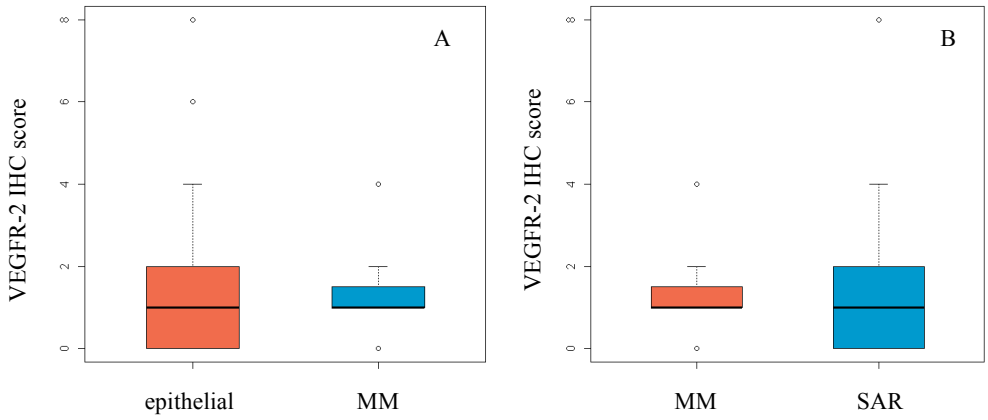


Figure 19: The VEGFR-2 IHC score in dogs with epithelial and MM (A), and MM and SAR (B). Mann-Whitney-Wilcoxon test shows no significant difference (p=0.741 and p=0.839, respectively).

Among epithelial head and neck tumours sino-nasal SCC were demonstrated with the highest percentage of expression of VEGFR-2. Significant difference in the expression of VEGFR-2 between oral SCC and SNSCC HN tumours, with SNSCC expressing the highest percentage of VEGFR-2 was confirmed ($p<0.001$) (Figure 20 A). The same was true for the VEGFR-2 IHC score when comparing oral SCC and SNSCC HN tumours (Figure 20 B). There was also the difference in the intensity of the expression of VEGFR-2. Oral SCC expressed no VEGFR-2 or the expression was mild while the intensity of the VEGFR-2 expression in SNSCC tumours was moderate or strong with all SNSCC tumours expressing VEGFR-2.

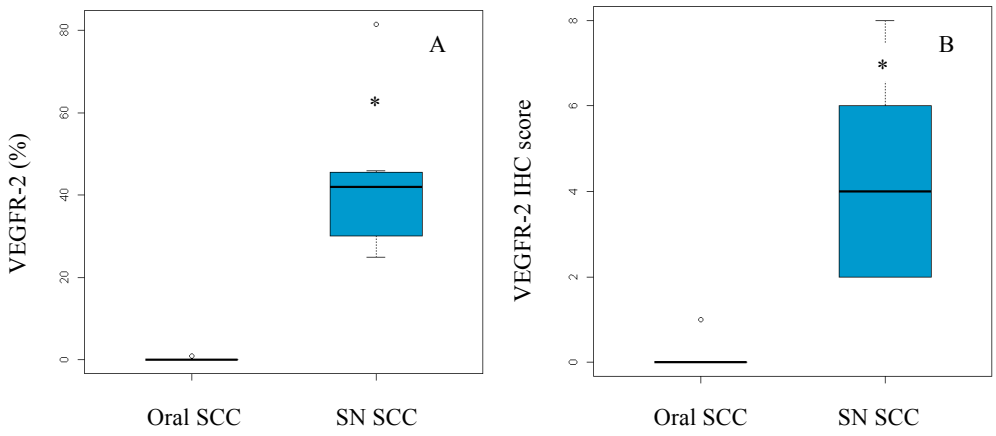
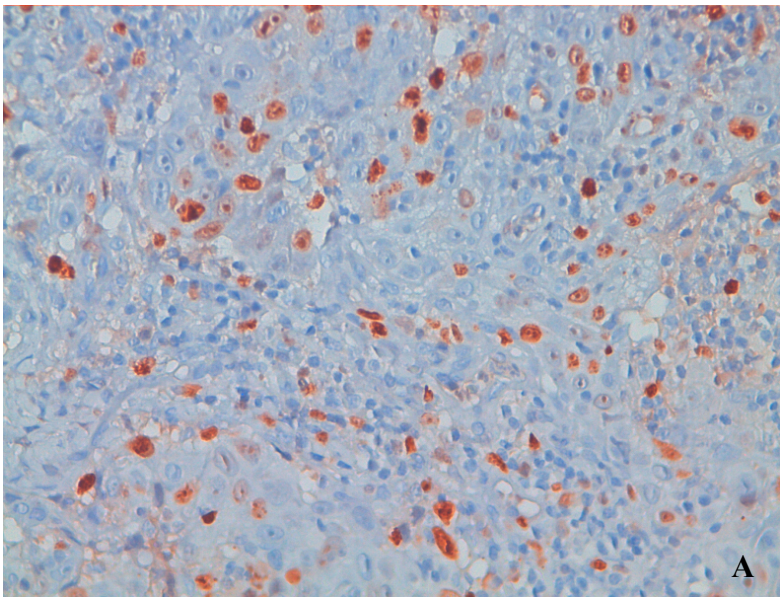
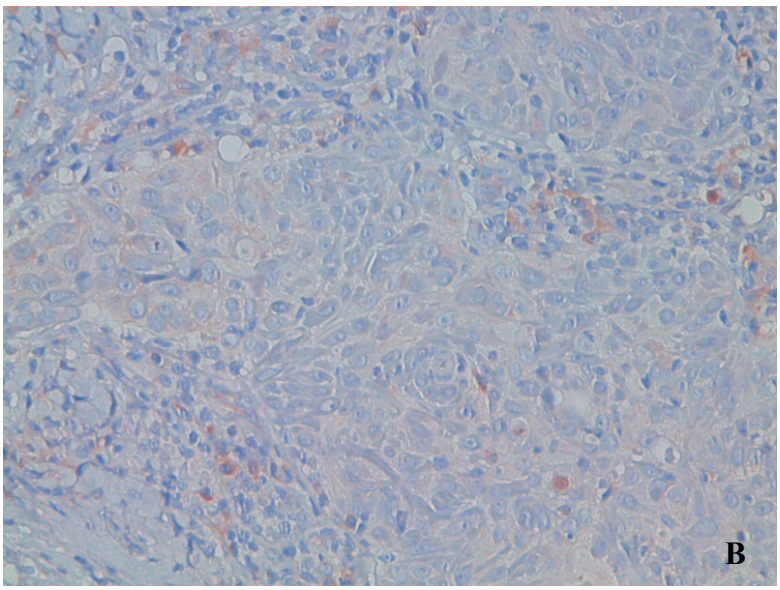


Figure 20: The percentage of expression of VEGFR-2 (A) and VEGFR-2 IHC score (B) in dogs with oral SCC and sinonasal SCC; Mann-Whitney-Wilcoxon test shows significant difference* ($p<0.001$ and $p=0.0003$, respectively).

High correlation between VEGFR-2 and Ki-67 in malignant HN conditions was confirmed ($r=0.65$; $p<0.0001$). Only weak positive correlation between Ki-67 and VEGFR-2 in oral SCC ($r=0.18$) was detected and the correlation was not statistically significant ($p=0.15$). Strong positive correlation between Ki-67 and VEGFR-2 in sino-nasal SCC ($r=0.84$; $p=0.0002$) but only moderate correlation in oral mesenchymal tumours was detected ($r=0.35$; $p=0.002$). According to the Spearman′s rank test the correlation between tumour stage and Ki-67 was high ($r=0.63$) while the correlation between mitotic count and necrosis with Ki-67 was moderate ($r=48$; $r=0.50$, respectively). Moderate correlation between tumour stage, mitotic count and necrosis with VEGFR-2 were detected ($r=0.55$, $r=0.31$, $r=0.44$, respectively). The differences were statistically significant ($p<0.05$). Strong negative correlation between Ki-67 and VEGFR-2 in inflammatory and benign oral conditions in dogs was detected.

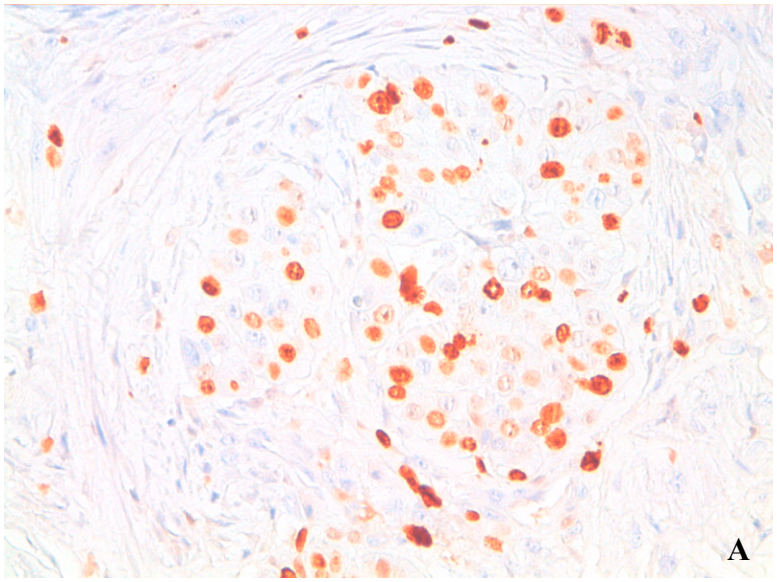


Ki-67 (40X)

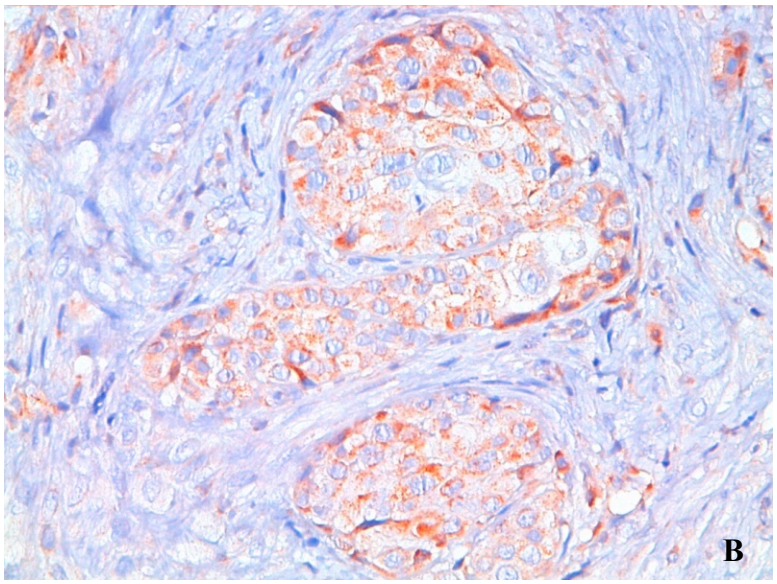


VEGFR-2 (40X)

Figure 21: Ki-67 (panel A) and VEGFR-2 (panel B) expression in canine oral SCC. Ki-67 positive cells (brown colour) (38%) are predominantly present in the periphery of the tumour nests and this was classified as moderate Ki-67 expression. Canine oral SCC was negative for VEGFR-2 expression (panel B).



Ki-67 (40X)



VEGFR-2 (40X)

Figure 22: Ki-67 (panel A) and VEGFR-2 (panel B) expression in canine sino-nasal SCC. Ki-67 positive cells (37.5%) are predominantly present in the periphery of the tumour nests. In the higher scoring tumours more cells towards the centre of the tumour nests exhibited positive VEGFR-2 labelling. High intensity tumour cell cytoplasmatic labelling for VEGFR-2 in canine sino-nasal SCC (brown coulour) (81.5%) was detected and this was classified as strong VEGFR-2 expression (panel B).

Table 16: Univariate statistical analysis of factors potentially associated with overall survival time (OS) in 34 dogs with malignant head and neck tumours.

Factor	Number of dogs	OS (days) median	HR	95% CI	P-value
Location					
Oral*	27 (79 %)	442	3.91	1.48 - 10.31	**0.006**
SN	7 (21 %)	280			
Histological type					
Epithelial t.*	17 (50 %)	692			
MM	7 (21 %)	354	1.41	0.49 - 4.05	0.530
SAR	10 (29 %)	366	1.85	0.75 - 4.53	0.180
Histological type					
Epithelial t.*	17 (50 %)	692	1.65	0.75 - 3.66	0.213
MM+SAR	17 (50 %)	376			
Stage					
I+II*	17 (50 %)	745	2.75	1.24 - 6.08	**0.013**
III+IV	17 (50 %)	280			
Mitotic count (n/10HPF)					
Low*	19 (56 %)	442	1.79	0.82 - 3.90	0.141
High	15 (44 %)	301			
Necrosis					
Yes	13 (38 %)	267	8.57	3.03 - 24.21	**<0.001**
No*	21 (62 %)	745			
Grade					
I+II* (low)	27 (79 %)	442	8.24	2.83 - 24.00	**<0.001**
III (high)	7 (21 %)	186			
Ki-67 IHC score					
Low (< 2)*	6 (18 %)	729	1.46	0.55 - 3.89	0.450
High (≥ 2)	28 (82 %)	386			
Ki-67 (%)					
Low (≤ 14.2)*	17 (50 %)	692	1.75	0.80 - 3.79	0.159
High (> 14.2)	17 (50 %)	280			
VEGFR-2 IHC score					
Low (< 1)*	13 (38 %)	784	6.25	2.23 - 17.50	**<0.001**
High (≥ 1)	21 (62 %)	301			
VEGFR-2 (%)					
Low (< 1)*	22 (65 %)	735	10.63	3.87 - 29.19	**<0.001**
High (≥1)	12 (35 %)	237			

(*referent category)

6.3.4 Kaplan-Meier curves for overall survival in 34 dogs with malignant head and neck tumours according to the variables investigated

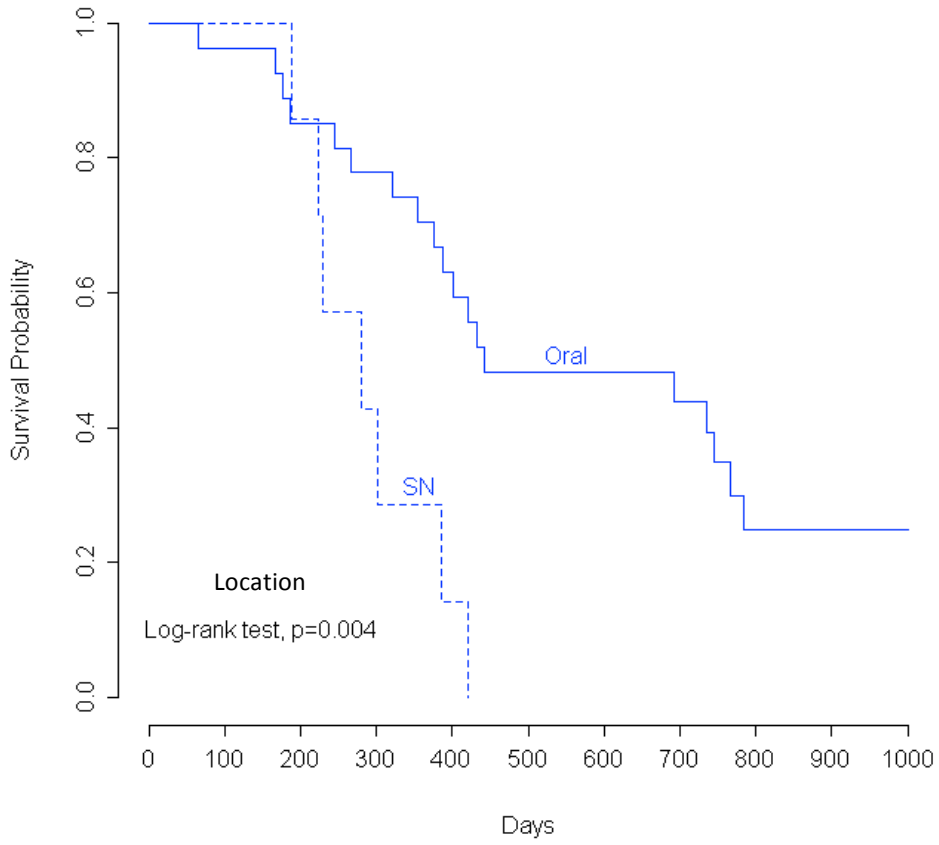


Figure 23: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to the location (oral *vs* sino-nasal) in dogs with malignant head and neck tumours. Significantly shorter survival rates of dogs with sino-nasal head and neck tumours in comparison with dogs with oral head and neck tumours were detected (p=0.004).

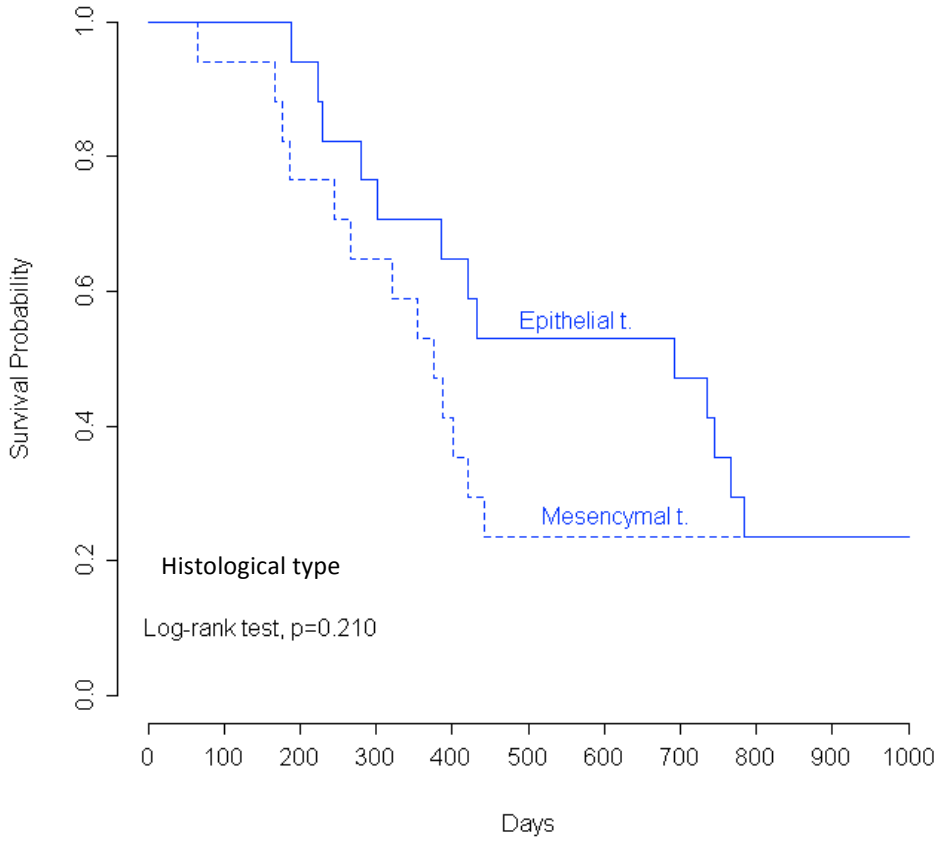


Figure 24: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to the histological type (epithelial *vs* mesenchymal-consisting of malignant melanoma and sarcoma tumours) in dogs with malignant head and neck tumours. Statistical trend towards shortened survival rates in dogs with malignant melanoma and sarcoma tumours in comparison with dogs with epithelial head and neck tumours was detected. The difference was not statistically significant (p=0.210).

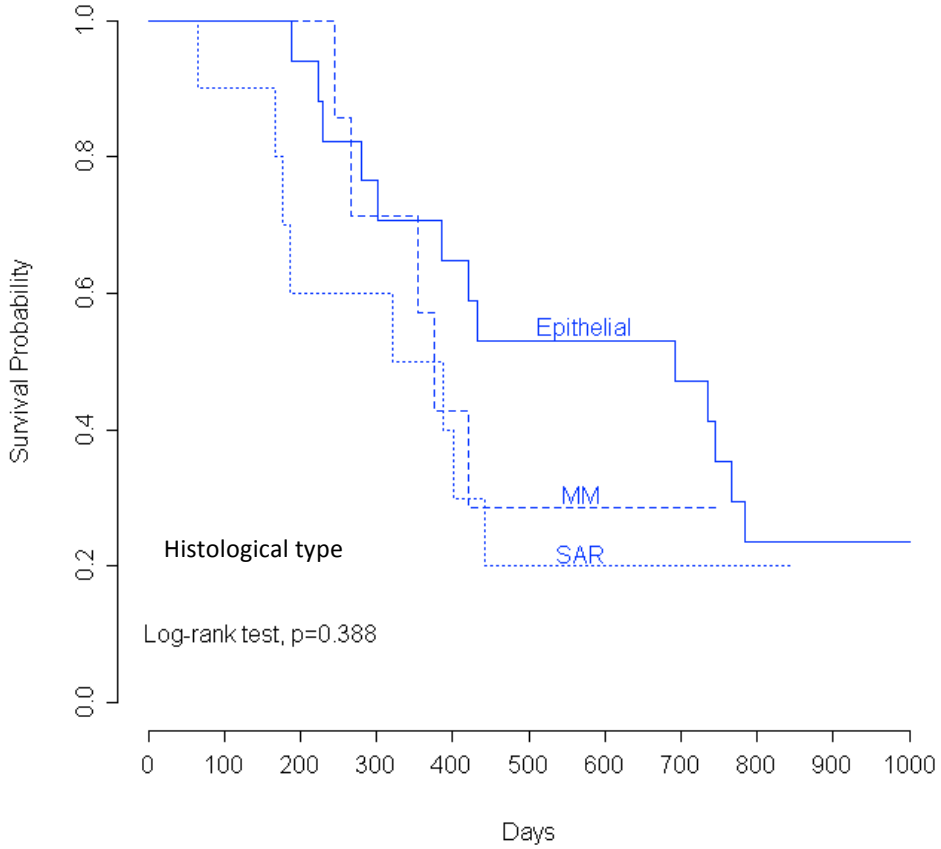


Figure 25: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to the histological type (epithelial *vs* MM *vs* SAR) in dogs with malignant head and neck tumours. Statistical trend towards shortened survival rates in dogs with MM and SAR head and neck tumours in comparison with dogs with epithelial head and neck tumours was detected. The difference was not statistically significant (p=0.388).

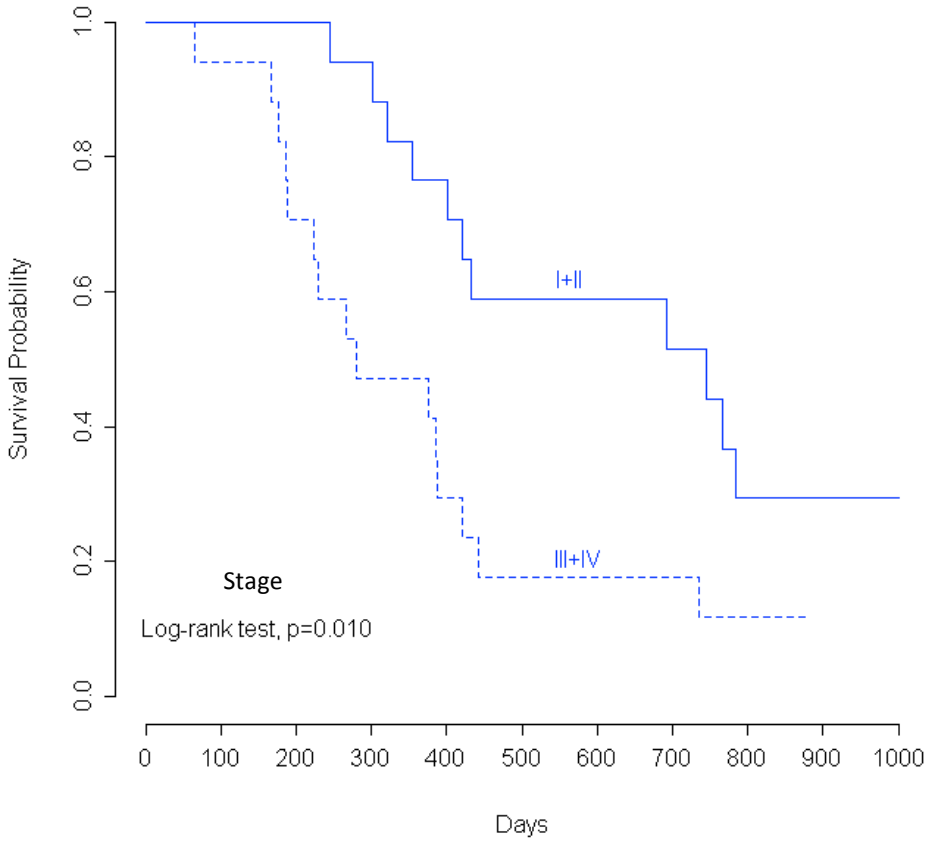


Figure 26: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to the stage (stage I+II *vs* stage III+IV) in dogs with malignant head and neck tumours. Significantly increased survival rates of dogs with stage I+II (n=17) in comparison with dogs with stage III+IV malignant head and neck tumours (n=17) was detected (p=0.010).

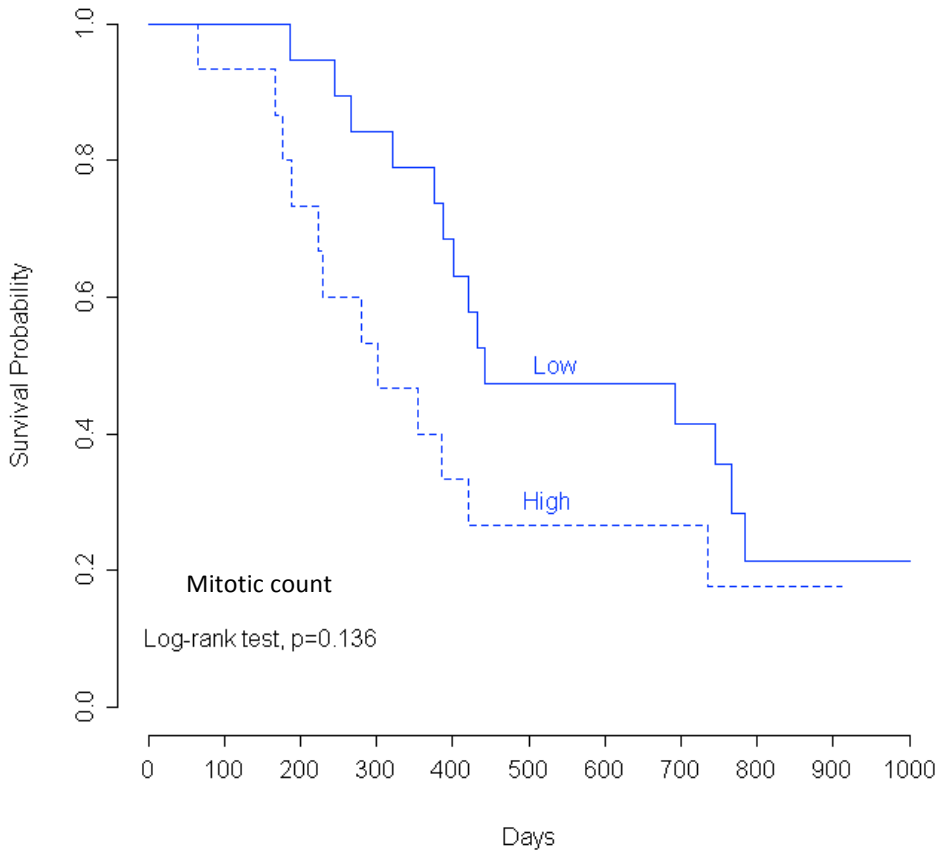


Figure 27: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to mitotic count (low *vs* high) in dogs with malignant head and neck tumours. A trend towards increased survival rates in dogs with low mitotic count in comparison with dogs with high mitotic count was detected. The difference between groups was not statistically significant (p=0.136).

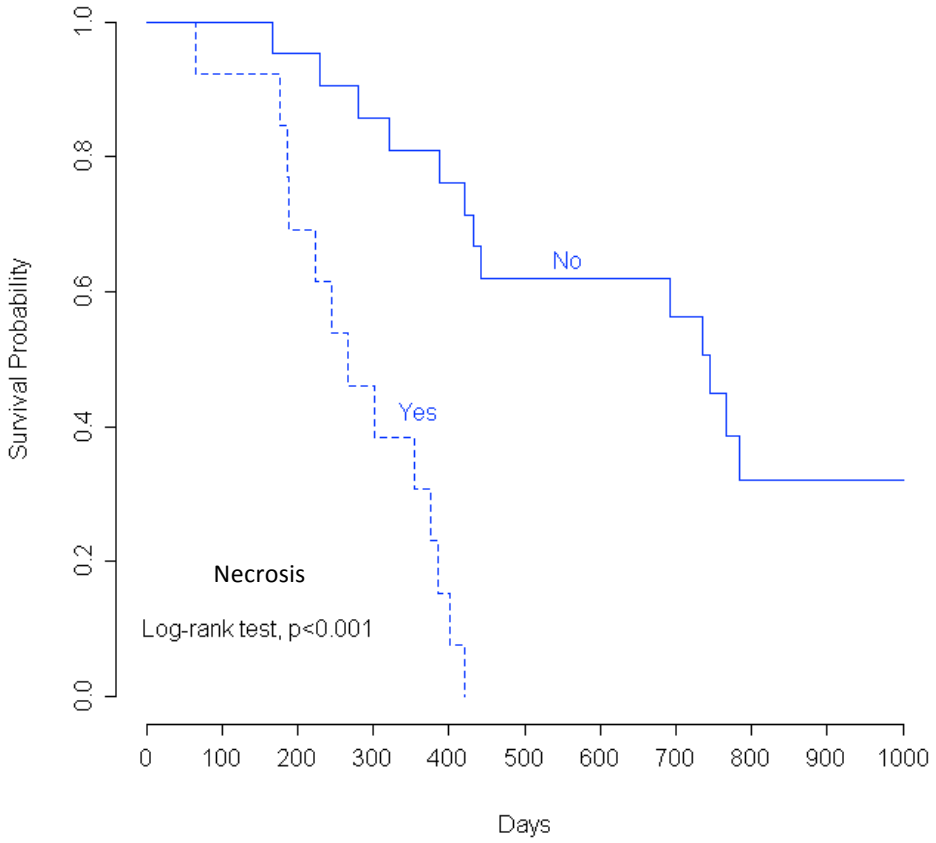


Figure 28: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to the presence of necrosis (necrosis yes *vs* necrosis no) in dogs with malignant head and neck tumours. Significantly increased survival rates were detected in dogs with tumours with no necrosis in comparison with dogs with malignant head and neck tumours where necrosis was present ($p<0.001$).

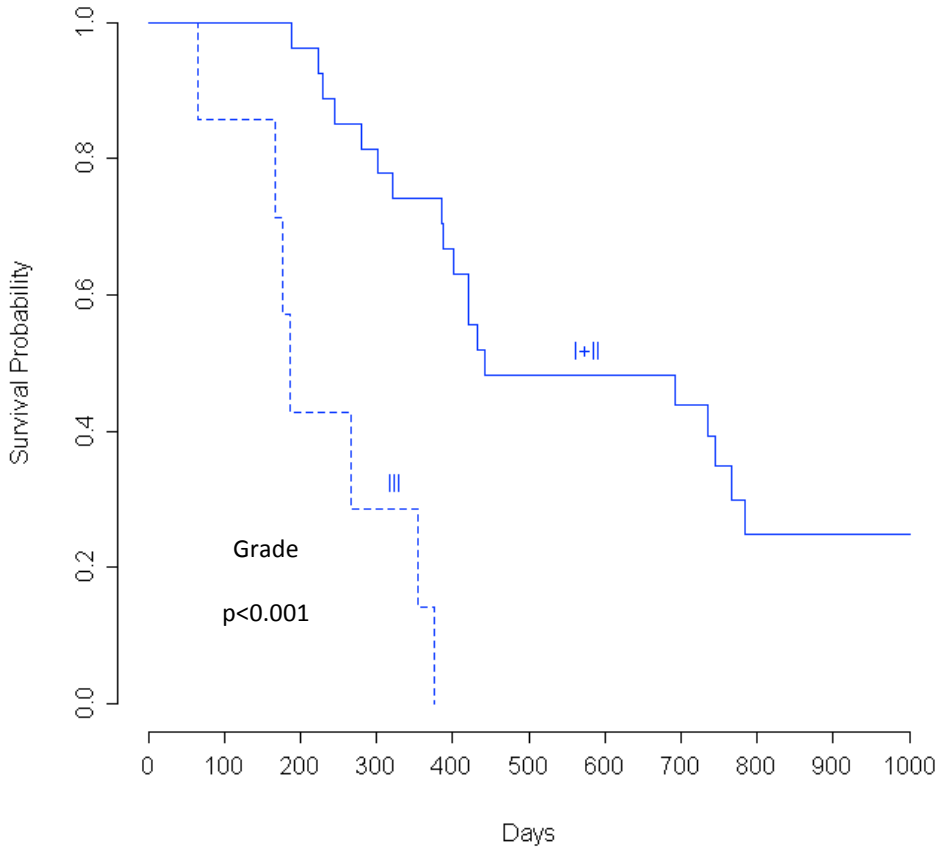


Figure 29: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to tumour grade in dogs with malignant head and neck tumours. Significantly shorter survival rates in dogs with malignant head and neck tumours with high grade (grade III) in comparison with dogs with HN tumours with low grade (grade I+II) were detected ($p<0.001$).

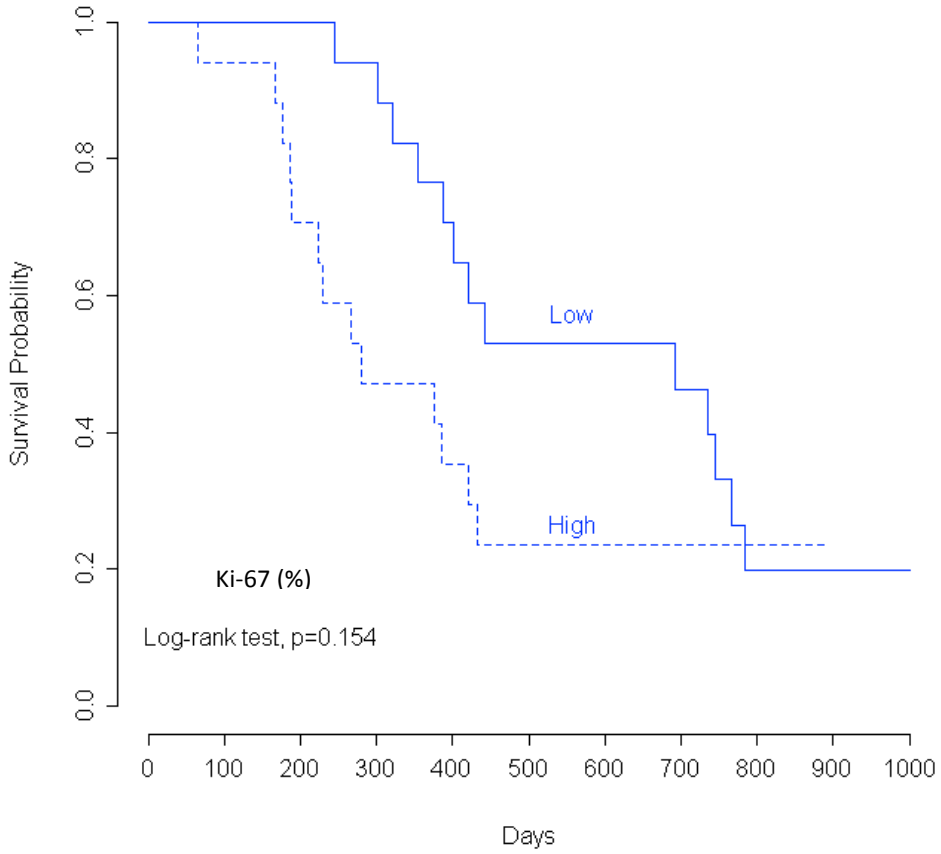


Figure 30: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to the expression of Ki-67 (low *vs* high %) in dogs with malignant head and neck tumours. Statistical trend towards shortened survival rates in dogs with malignant head and neck tumours with high Ki-67 expression in comparison with dogs with malignant head and neck tumours with low Ki-67 expression was detected. The difference between groups was not statistically significant (p=0.154).

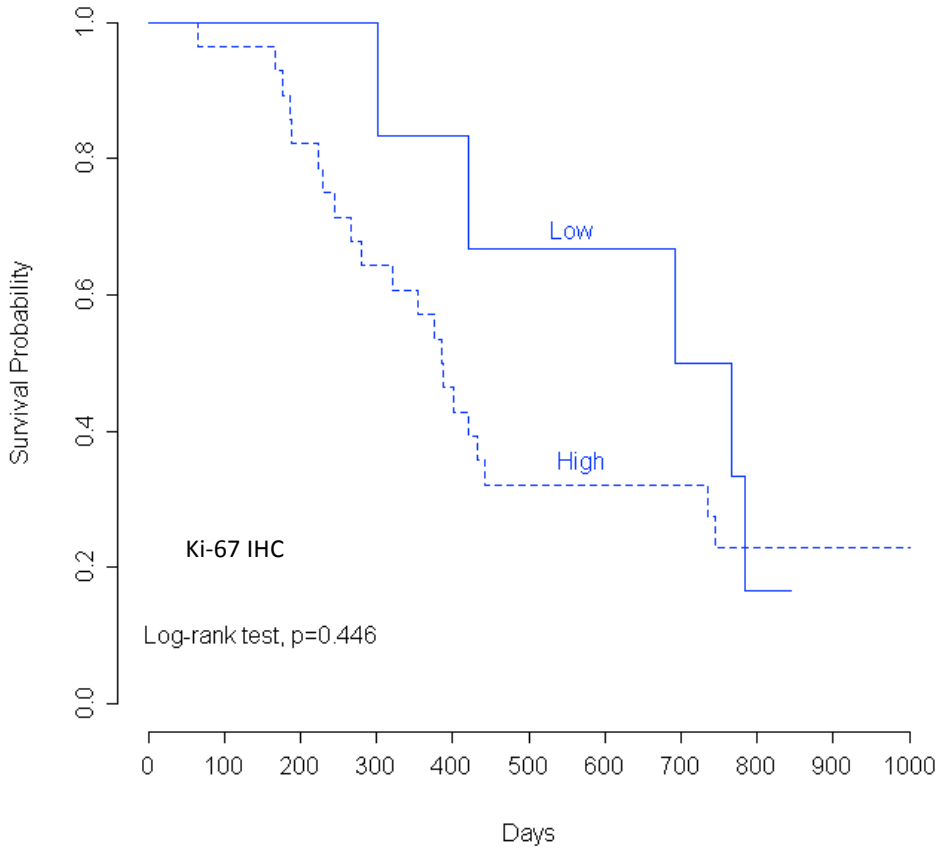


Figure 31: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to Ki-67 IHC score (low *vs* high) in dogs with malignant head and neck tumours. A trend towards shortened survival rates in dogs with malignant head and neck tumours with high Ki-67 IHC score in comparison with dogs with malignant head and neck tumours with low Ki-67 IHC score was detected. The difference between groups was not statistically significant (p=0.446).

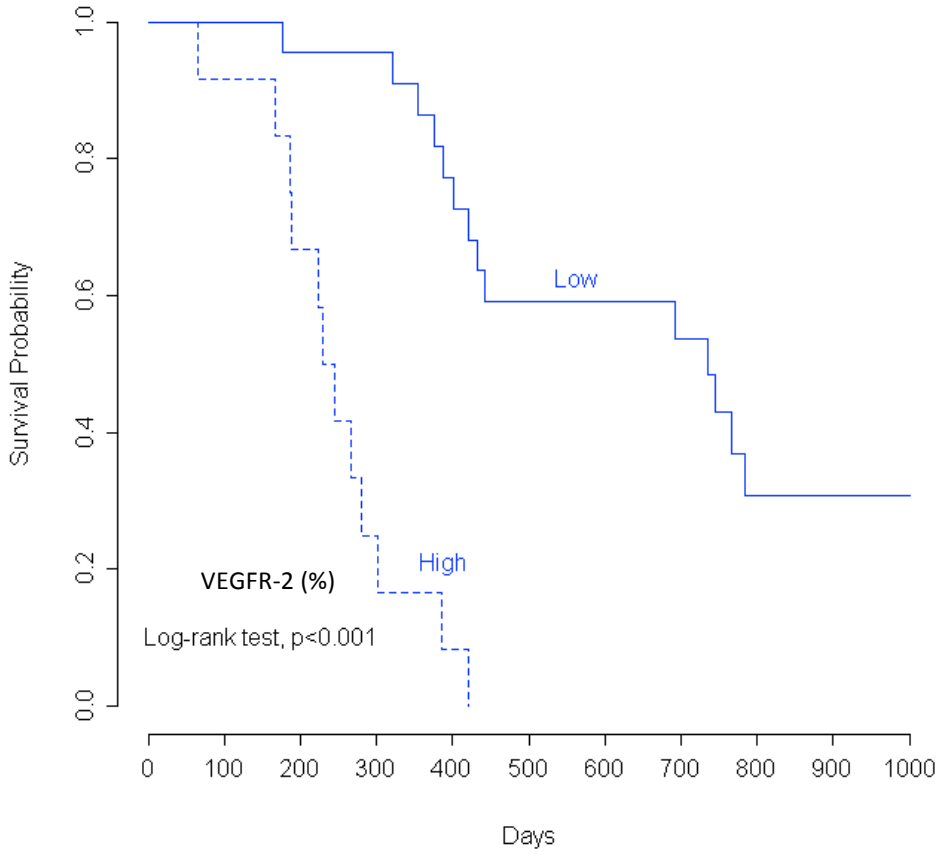


Figure 32: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to VEGFR-2 expression (low *vs* high percentage) in dogs with malignant head and neck tumours. Significantly shorter survival rates in dogs with malignant head and neck tumours with high VEGFR-2 expression in comparison with dogs with malignant head and neck tumours with low VEGFR-2 expression were detected ($p<0.001$).

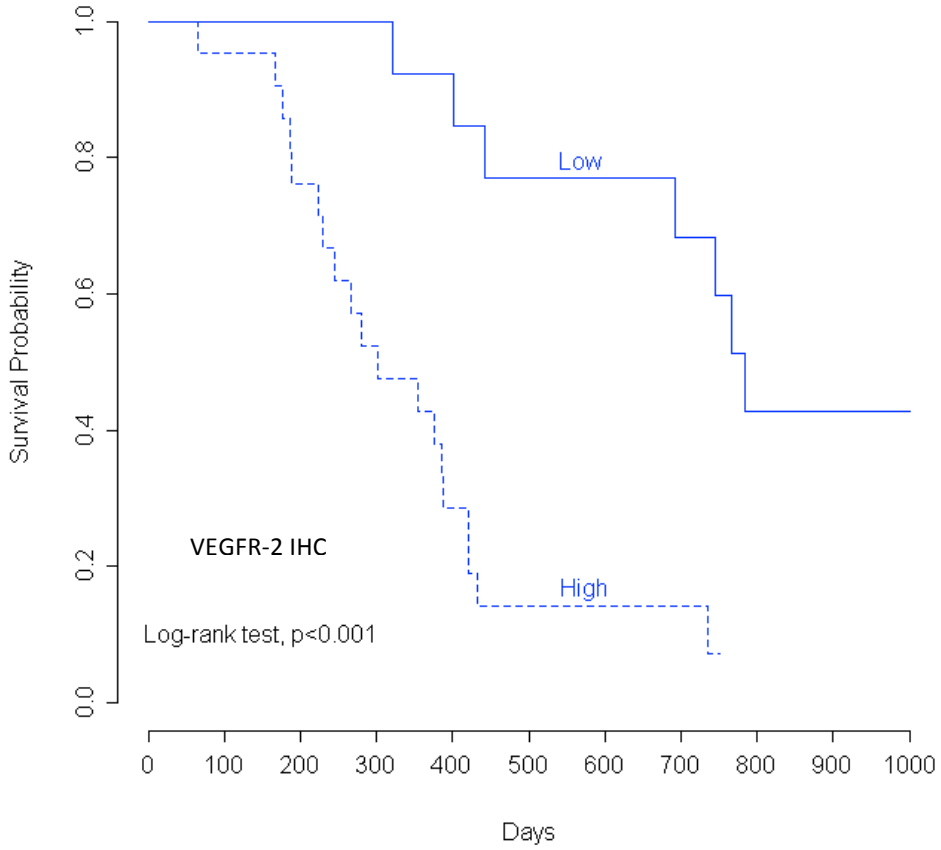


Figure 33: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to VEGFR-2 IHC score (low *vs* high) in dogs with malignant head and neck tumours. Significantly shorter survival rates in dogs with malignant head and neck tumours with high VEGFR-2 IHC score in comparison with dogs with malignant head and neck tumours with low VEGFR-2 IHC score were detected ($p<0.001$)

Table 17: Univariate analysis of prognostic value of the percentage and IHC score of Ki-67 and VEGFR-2 in 54 dogs with inflammatory, benign and malignant head and neck conditions.

Factor	Number of dogs	OS (days) Median	HR	95% CI	P-value
Ki-67 IHC score					
Low (< 2)*	22 (41 %)	/	4.82	1.80 - 12.90	0.002
High (≥ 2)	32 (59 %)	410			
Ki-67 (%)					
Low (≤ 7.9)*	27 (50 %)	/	3.71	1.60 - 8.60	0.002
High (> 7.9)	27 (50 %)	420			
VEGFR-2 IHC score					
Low (< 1)*	33 (61 %)	/	17.4	6.13 - 49.60	<0.001
High (≥ 1)	21 (39 %)	301			
VEGFR-2 (%)					
Low (< 1)*	34 (63 %)	/	13.43	5.03 - 35.90	<0.001
High (≥ 1)	20 (37 %)	328			

*Referent category

6.3.5 Kaplan-Meier curves for overall survival in 54 dogs with inflammatory, benign and malignant head and neck tumours according to Ki-67 and VEGFR-2 expression

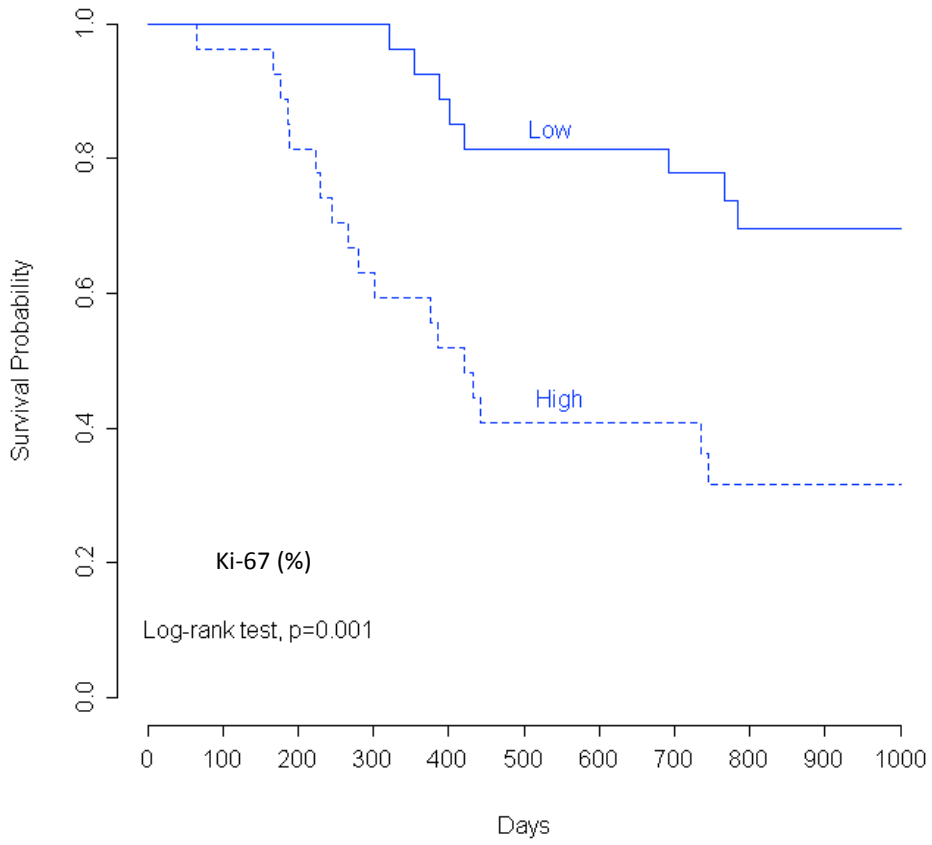


Figure 34: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to the Ki-67 expression (low *vs* high percentage) in dogs with inflammatory, benign and malignant HN conditions. Significantly increased survival rates of dogs with inflammatory, benign and malignant head and neck tumours with low Ki-67 expression in comparison with dogs with inflammatory, benign and malignant head and neck tumours with high Ki-67 expression were detected (p=0.001).

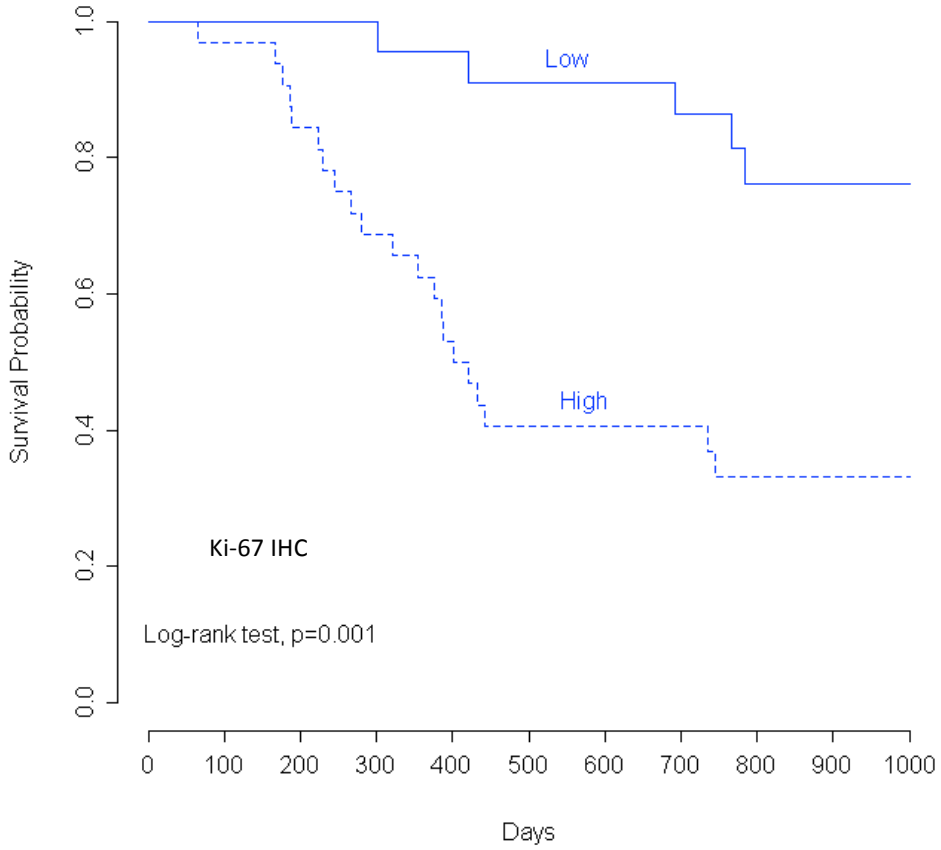


Figure 35: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to the Ki-67 IHC score (low *vs* high) in dogs with inflammatory, benign and malignant head and neck tumours. Significantly increased survival rates of dogs with inflammatory, benign and malignant HN conditions with low Ki-67 IHC score in comparison with dogs with inflammatory, benign and malignant head and neck tumours with high Ki-67 expression were detected (p=0.001).

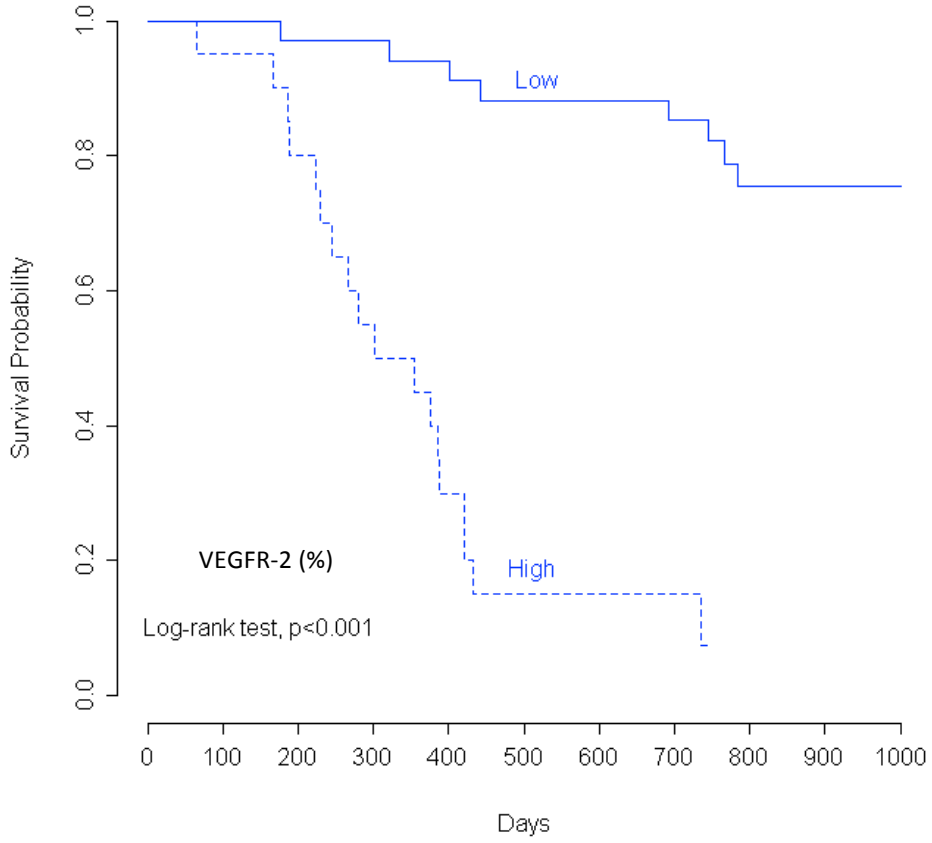


Figure 36: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to VEGFR-2 expression (low *vs* high percentage) in dogs with inflammatory, benign and malignant head and neck conditions. Significantly increased survival rates of dogs with inflammatory, benign and malignant head and neck tumours with low VEGFR-2 expression in comparison with dogs with inflammatory, benign and malignant head and neck tumours with high VEGFR-2 expression were detected ($p<0.001$).

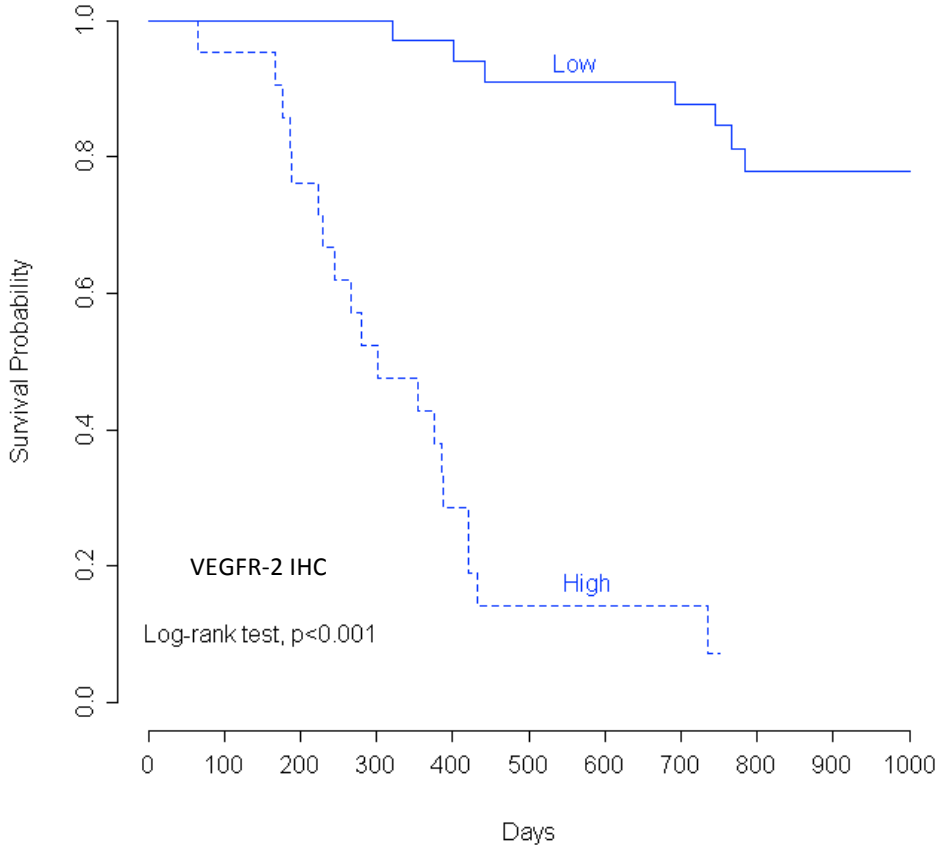


Figure 37: Kaplan-Meier survival curves (Log-rank, Cox model univariate) for overall survival according to VEGFR-2 IHC score (low *vs* high) in dogs with inflammatory, benign and malignant head and neck conditions. Significantly increased survival rates of dogs with inflammatory, benign and malignant head and neck tumours with low VEGFR-2 IHC score in comparison with dogs with inflammatory, benign and malignant head and neck tumours with high VEGFR-2 IHC score were detected ($p<0.001$).

6.4 Accelerated chemoradiotherapy protocol for the treatment of advanced canine head and neck squamous cell carcinoma

6.4.1 Patients

One spayed female, three neutered and three intact males were included in the study. The breeds included two mixed breed dogs, one Labrador Retriever, one Golden Retriever, one Polish Lowland Sheepdog, one Foxterier and one poodle. The mean age at the presentation was 11.8 years (range 8-16 years). The dogs were presented with varying clinical signs of different duration that included dyspnoea (n=4), halitosis (n=3), salivation (n=2), dysphagia (n=2), pain (n=1) and exercise intolerance (n=1), epistaxis (n=4), haemorrhagic/mucopurulent nasal discharge (n=4), sneezing (n=4), facial deformity (n=4), exophthalmos (n=3) and neurologic signs (n=1). Two dogs were already diagnosed with rostral mandibular SCC and sinonasal SCC and were referred for further evaluation and treatment. No other previous problems were reported in the history.

All dogs were in normal body condition with normal heart and lung sounds. Bilateral mild masticatory muscle wastage was noticed in two dogs with oral/tonsillar SCC. Pain was elicited on attempts to open the mouth in one dog oral/tonsillar SCC. Dogs with SNSCC and a dog with nasal planum SCC were presented with facial deformity. Regional lymph nodes were normal in size and consistency on palpation in six dogs while a unilateral mandibular lymphoadenomegaly (7 x 8.3 x 4.2 cm) was detected in one dog oral/tonsillar SCC. Solid masses arising from the right tonsil (n=1), the rostral mandibular gingiva (n=1) and an ulcerative, bleeding gingival proliferation extending from the third premolar tooth (107) to the end of the right maxilla (n=1) were detected in dogs with oral/tonsillar SCC. Computed tomography (CT) (n=7) in dogs with advanced HNSCC and dental radiography in dogs with oral SCC showed local maxillary, mandibular and rostral mandibular bone lysis in dogs

with oral/tonsillar SCC and destruction of maxillary, nasal and frontal bones with cribriform involvement with abnormal soft tissue density in dogs with SNSCC. Dogs with oral SCC had stage 1,2 and a dog with tonsillar SCC had stage 3. CT confirmed a severe mandibular and retropharyngeal lymphadenomegaly in the tonsillar SCC case. Dogs with oral/tonsillar SCC were stage 1-3 while all dogs with SNSCC were stage 4. Dog with nasal planum SCC had stage 2. Abdominal cavity ultrasonography (n=7) and thorax CT (n=7) were unremarkable in all seven dogs and did not show any evidence of distant metastatic disease at the time of presentation.

For confirmation of the tumour type, incisional biopsies from the primary tumour site were obtained surgically under general anaesthesia in 2 dogs with the oral/tonsillar caudal lesions. The third case with the rostral mandibular mass was already diagnosed at the time of presentation. Nasal biopsies were performed in three dogs with sino-nasal tumours and an incisional biopsy in a dog with nasal planum mass. Fine needle regional lymph node aspirates were performed in all cases at the time of evaluation and were submitted for cytological examination for staging purposes. All seven dogs had a histopathological diagnosis of SCC. No lymph node involvement was confirmed based on cytological examination of the regional lymph node aspirates in six dogs while the dog with the tonsillar mass was diagnosed with regional lymph node metastasis, as suspected on CT.

6.4.2 Radiation therapy

All dogs completed the radiation therapy protocol as planned and no effects related to twice-daily anaesthesia were observed. Rapid partial response to therapy was seen in all dogs by the end of the 9-day protocol. 6-10 weeks after completion of therapy all three dogs with oral/tonsillar SCC had achieved a complete response.

The partial response in 3 dogs with advanced SNSCC was seen 2-3 months after finished radiotherapy according to a CT examination. One year after finished radiotherapy two dogs with SNSCC achieved a complete response while the response in one dog with SNSCC was assessed as partial. After that, no CT examination was performed in dogs with SNSCC, but the dogs were, according to the referring veterinarians, without visible signs of tumour progression after that period of time. The same is true for the dog with SNSCC that is still alive. The referring veterinarian was contacted and advised to suggest the owners to perform a CT for evaluation of the response. A dog with nasal planum SCC achieved only a partial response and the tumour progressed 6.5 months after finished radiotherapy treatment.

6.4.3 Toxicity

The most significant acute toxicity occurred in the mucosal lining (mild to strong soft tissue reactions and local swellings) in dogs with oral and tonsillar SCC. Only a dog with rostral oral SCC experienced mild soft tissue reaction while the other two dogs (dogs with maxillary and a dog with tonsillar SCC) experienced stronger mucosal acute reactions assessed as grade 3. Grade 3 acute side effects in dogs with SNSCC and nasal planum SCC were detected in all 3 dogs. The acute side effects resolved quickly with a course of antibiotics (amoxicillin clavulanic acid and metronidazole) and non-steroidal anti-inflammatory drugs (carprofen or meloxicam) in all dogs with advanced head and neck SCC. The loss of mucosal integrity associated with bone exposure became evident after 4 days of radiotherapy due to the early tumour response in a dog with maxillary SCC and healed by second intention 2 months after completion of radiation treatment. The cutaneous toxicity ranged from grade 2 to grade 3. Otitis externa-media was detected in 2 dogs and resolved within 3 weeks with antibiotic therapy (enrofloxacine 7 mg/kg 24 hrs 2 weeks). Late toxicity effects could be evaluated for all dogs and included alopecia (n=7) or leukotrichia (n=6) within the radiation field, which generally developed 4-6 months post irradiation. No late side effects to the bone, confirmed by intraoral dental radiography and computed

tomography, every 6 months up to 1-3 years were seen. All dogs with SNSCC developed cataract because the eye affected with the tumour was included in the radiation filed. No owner felt that therapy had negative effect on theirs dog's quality of life. All three dogs had improvement in eating and drinking and gained weight 2-3 months after completing radiotherapy.

6.4.4 Follow-up

The dog with a stage I rostral mandibular SCC is still alive at 1155 days after commencing the radiotherapy with no signs of tumour recurrence or evidence of local or distant metastatic disease.

The dog with a stage II maxillary SCC (Figure 38) was euthanized due to cardiac failure 919 days after commencing the radiotherapy course with the primary site in a complete remission and no evidence of the distant metastatic disease.

The dog with a stage III tonsillar SCC (Figure 39) experienced local tumour recurrence in the irradiated ipsilateral regional lymph node 752 days (TTP 752 days) after commencing the radiotherapy protocol. There was no evidence of distant metastatic disease confirmed on CT. The dog was euthanized due to regional lymph node tumour progression 844 days after commencing the radiotherapy protocol due to inappetence and dysphagia. There was no evidence of tumour recurrence in the right tonsil.

Two dogs with stage 4 SNSCC died due to tumour non-related causes 489 and 396 days after commencing the radiotherapy treatment. According to the referring

veterinarian reports there were no visible signs of tumour progression in both of the dogs at the time of death and both dogs died due to tumour non-related causes. One dog with stage 4 SNSCC is still alive with no visible signs of tumour progression (426 days after commencing the radiotherapy treatment). The dog with nasal planum SCC (stage 2) was euthanized 246 days after commencing the radiotherapy treatment due to tumour progression. TTP in this case was 201 days.

Table 18: Characteristics of canine patients treated with an accelerated chemoradiotherapy protocol

nr.	BREED	AGE	SEX	WEIGHT	TUMOUR SITE	DURATION	TS	TR	TTP	OS
1	mixed breed	13	FN	12.8	right caudal maxilla	2 months	T2bM0M0	CR	/	919 d
2	mixed breed	8	M	35.6	right tonsil	1 month	T3N3bMo	CR	752d	844 d
3	labrador retriever *	12	M	32.4	rostral mandible	1 month	T1NoMo	CR (#)	/	1155d
4	Polish lowland sheep dog	13	MN	29.5	sinonasal	1.5 month	T4NoMo	PR	/	489 d
5	Foxterier *	8	M	16.8	sinonasal	3 months	T4NoMo	CR (#)	/	426 d
6	Poodle	16	MN	7.8	sinonasal	5 months	T4NoMo	CR/euth.	/	396 d
7	Golden retriever	13	MN	35.8	nasal planum	1 month	T2NoMo	PR	201d	246 d

Legend: CR: complete remission; PR: partial remission; Euth: euthanized due to other systemic diseases; *Referred with already diagnosed oral/tonsillar SCC; # Indicated still alive and tumour has not recurred or progressed at date of analysis; F: female; M: male; FN: female neutered; MN: male neutered; d = days; TS: tumour stage; TR: tumour response; TTP: time to progression; OS: overall survival

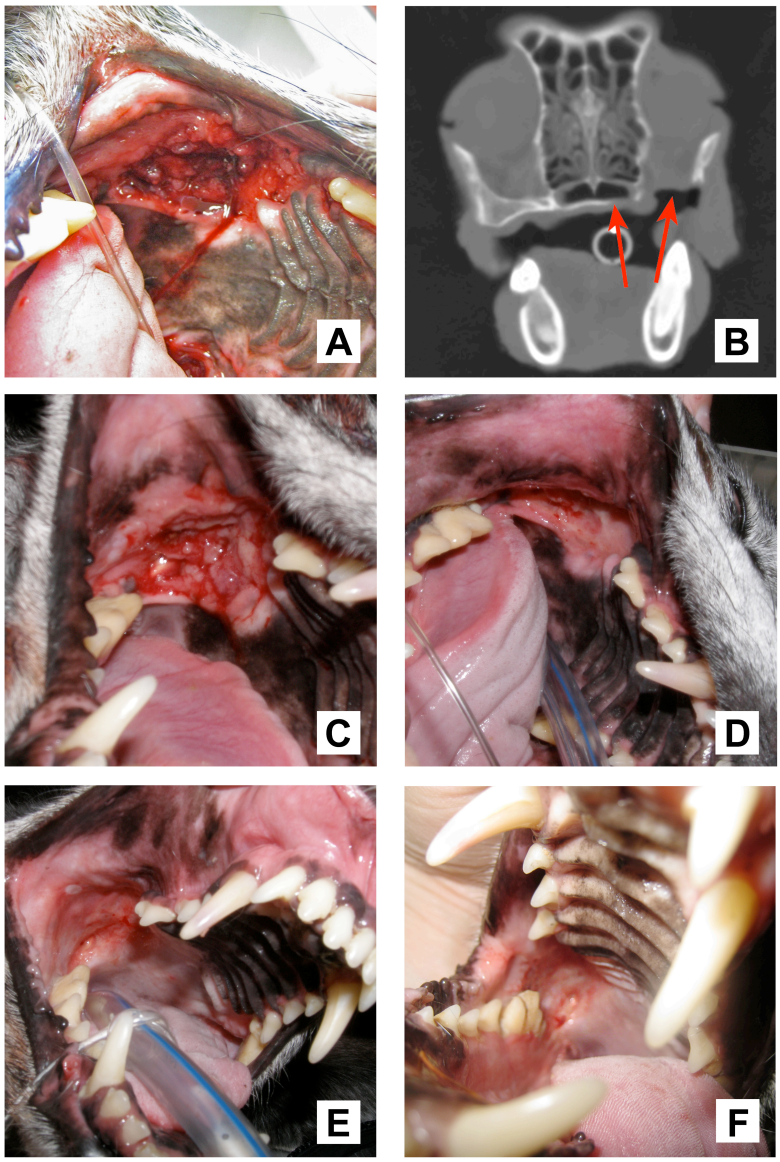


Figure 38: Ulcerative squamous cell carcinoma of the right maxillary region in a 13-year-old female mixed breed dog. (*Photo Rejec, A.)* A. The lesion as it appeared following the jaw manipulation required to perform a dental scaling and polishing during the diagnostic workup. B. CT scan showing bone loss associated with the lesion (arrows). C. Ulcerated appearance of the lesion after 5 days of the radiotherapy treatment. D. Three weeks after completion of therapy. There is significant mucositis but the lesion has shrunk significantly. E. Three months following treatment. The site is still healing. The site has a smooth surface with no evidence of tumour tissue. F. 24 months after treatment. The site still shows some erythema but the mucosa is smooth without any indication of neoplasia.

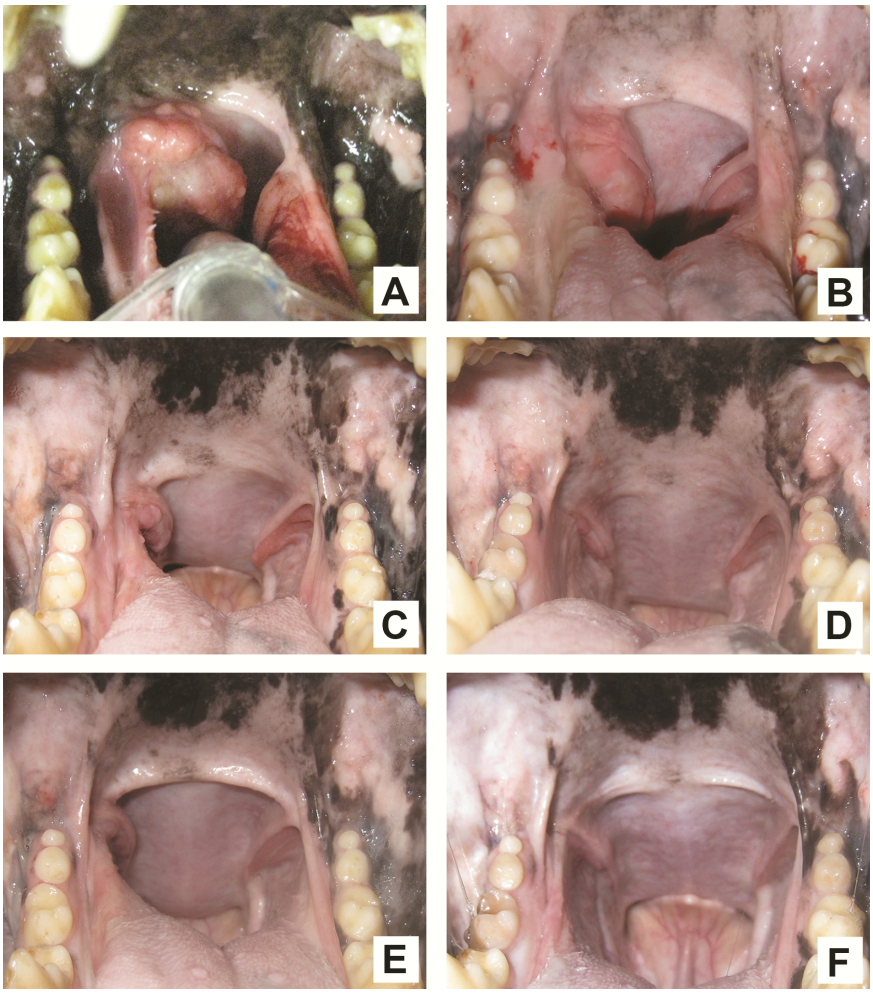


Figure 39: Tonsillar squamous cell carcinoma in an 8-year-old male mixed breed dog. (*Photo Rejec, A.)* A. Mass on presentation. B. Based on oral examinations, the lesion had subjectively shrunk to about a quarter of its original volume by the completion of the treatment. Note the loss of pigmentation due to the mucositis. C. Three weeks post treatment the mass is again much smaller. The mucosa has recovered the area. There is residual loss of pigmentation in the primary radiation field. D. Six weeks following treatment. The tonsil is almost back to normal. E. Eleven months following treatment. The two tonsils are similar in size; F. Two years following treatment. There is no evidence of tumour. Only a change in pigmentation remains

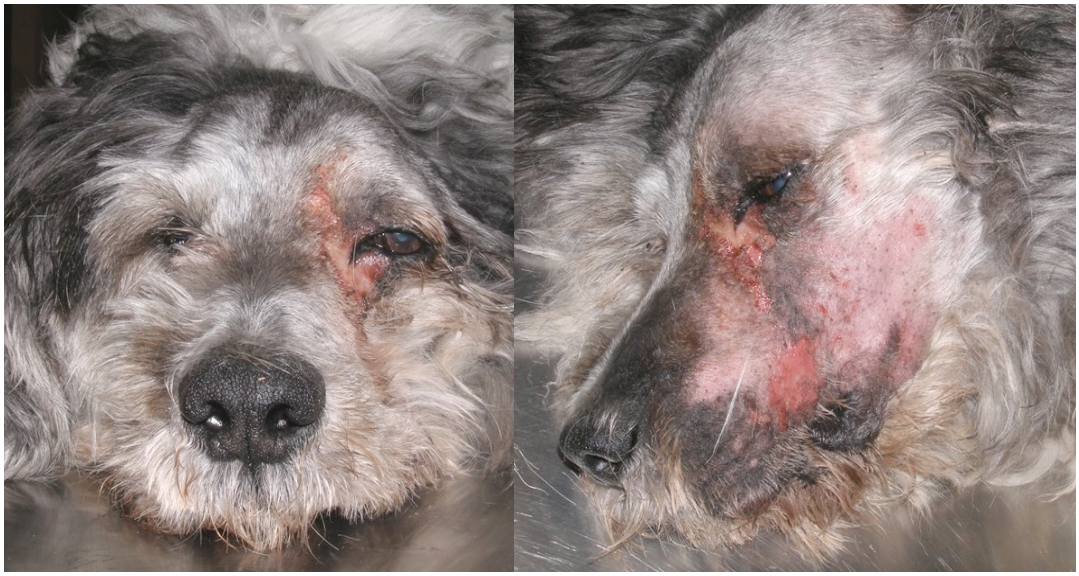


Figure 40: Sino-nasal SCC in a 13-year-old Polish Lowland Sheep dog 3 weeks after finished chemoradiotherapy protocol indicating acute radiotherapy side effects. (Photo Rejec, A.)

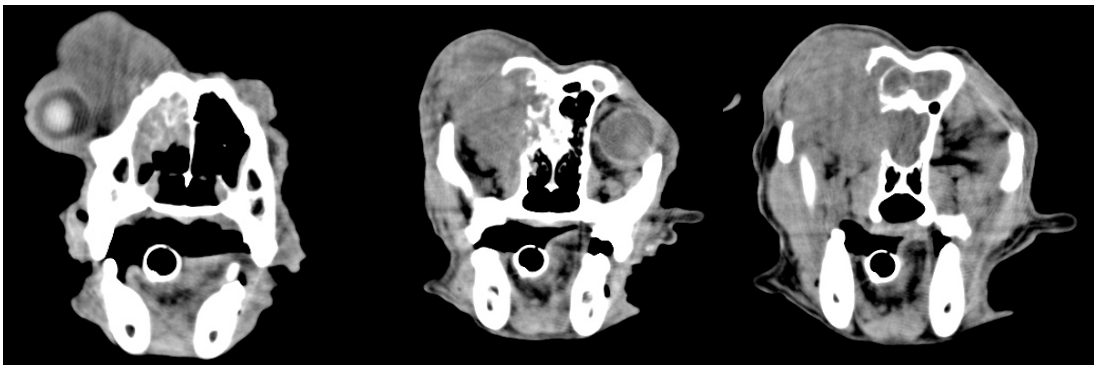


Figure 41: CT before commening chemoradiotherapy treatment (soft tissue window) in a 13-year-old Polish Lowland Sheep dog. Evidence of eye displacement and bone lysis due to tumour mass.

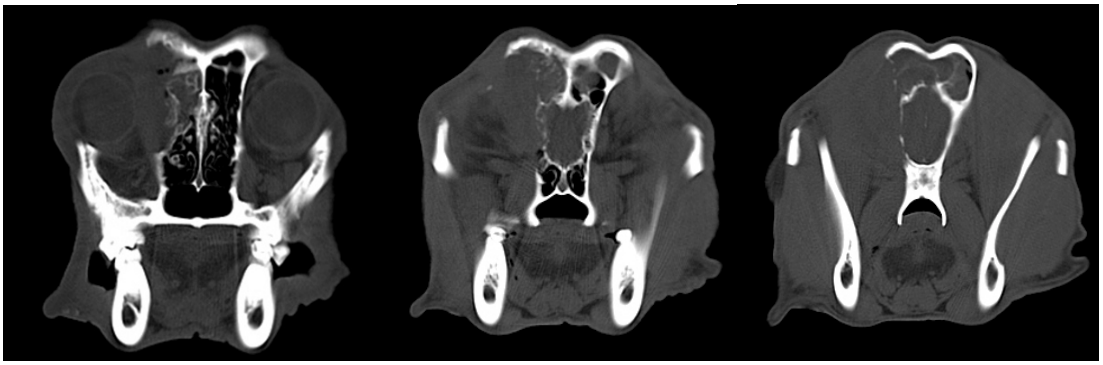


Figure 42: CT 2 months after finished chemoradiotherapy treatment (bone window) in a 13-year-old Polish Lowland Sheep dog showing a partial response. Eye turned to normal position. Bone defects are still present.

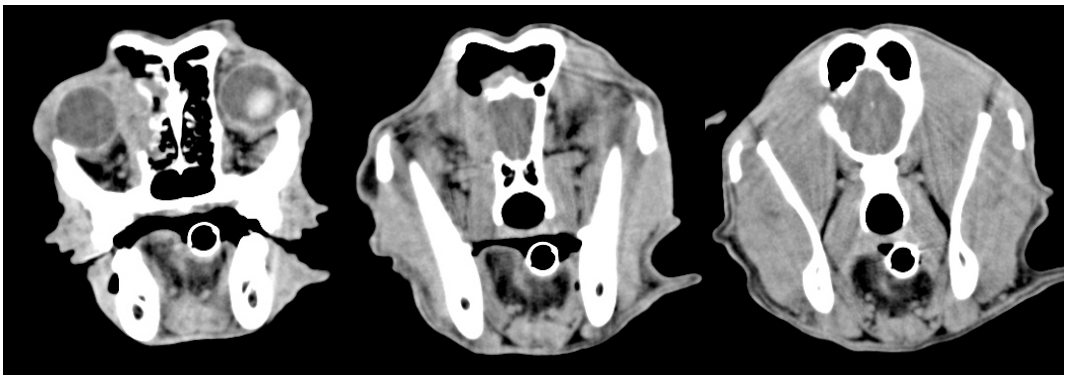


Figure 43: CT 1 year after finished chemoradiotherapy treatment (soft tissue window) in a 13-year-old Polish Lowland Sheep dog showing a partial response. Eye turned to normal position. Bone defects are still present.

Figure 44: 13-year-old Polish Lowland Sheep dog 4 months, 9 months and 14 months (with the left eye removed; last picture) after finished chemoradiotherapy protocol. The left eye was removed due to radiotherapy-induced damage. *(Photo Rejec, A.)*

7 DISCUSSION

A wide variety of clinically recognisable lesions of inflammatory, benign or malignant origin affect dog's head and neck region. The development and progression of the either conditions are determined by many factors associated with inflammation and/or neoplasm and the host (Arzi & Verstraete, 2012). Their biological behaviour is different as well as treatment planning strategies and prognosis. Although the treatment of inflammatory and benign lesions, for instance, PD and benign head and neck tumours in contrast with malignant ones, is quite straightforward and there is no major risk of the spread of the disease to distant sites, a lot of questions regarding the systemic effects, pathogenesis and treatment strategies of especially malignant head and neck conditions in dogs still remain unclear and open. Understanding the biological behaviour of those conditions exerting both local and systemic effects in the host, is an important fact as it enables the clinician to select the methods of treatment indicated more precisely and to inform the client timely and correctly (Arzi & Verstraete, 2012). Systemic effects have been described for both inflammatory and neoplastic head and neck conditions although with variable extent and in different patterns. More is known regarding the malignancy associated systemic inflammatory response that has been already shown to correlate with the pathogenesis and prognosis of certain cancers (Laird et al., 2013; Roxburgh & McMillan, 2014). What considers the PD, the data regarding its systemic effects and the cause-effect relationship are still debatable. As clinicians, we often observe that differences in the CBC parameters of dogs affected with PD or dogs with head and neck tumours exist. An interesting observation is that even dogs with advanced stages of PD most frequently have normal and very rarely changed values of CBC parameters, which is the opposite what we observe in dogs with malignant head and neck tumours.

Therefore, it was of our interest to investigate the differences between complete blood count (CBC) parameters and CBC indices in one of the most prevalent canine chronic inflammatory diseases, periodontal disease (PD), and neoplastic conditions

affecting dog's oral/pharyngeal and sino-nasal region and to elucidate the importance of these biomarkers in assessing the degree of inflammation, more precisely systemic inflammatory response, which is being closely associated with the development of both conditions. According to the author′s knowledge, there are no literature data estimating CBC parameters and calculated CBC indices (N/L, P/L, MPV/PLT, PLCRi) in a correlation with head and neck inflammatory and neoplastic conditions in dogs.

We characterized the role of CBC parameters and CBC indices on three groups of dogs (healthy dogs, dogs with stage 3,4 PD (moderate to advanced periodontitis) and dogs with neoplastic head and neck conditions) that did not suffer from any other concurrent inflammatory, immune-mediated or/and neoplastic diseases, which could have and effect on the parameters investigated.

Firstly, we hypothesised that CBC biomarkers, more specifically, CBC indices (N/L, P/L, MPV/PLT, PLCRi) differ and may thus serve as supportive distinguishing biomarkers between inflammatory and neoplastic head and neck conditions (NHNC) in dogs. As several studies from both human and veterinary medicine have shown that biomarkers of tumour associated systemic inflammatory responses have also an important effect on disease-related outcomes for many tumour types of different anatomical locations (Guthrie et al., 2013; McMillan, 2009), this aspect was assessed as well.

Inflammatory and neoplastic conditions interact directly and indirectly with host′s inflammatory cells (Elinav et al., 2013). In our study, the differences between dogs with stage 3,4 PD and NHNC in the frequency of circulating neutrophils and lymphocytes and N/L ratio were detected. Reduced number of lymphocytes was more pronounced in dogs with NHNC, which is in agreement with literature data indicating frequent reduction of lymphocyte number or occurrence of lymphopenia in cancer patients that develops as a consequence of the changes in the way the lymphocytes are produced, or are lost or destroyed (Mutz et al., 2013), although inflammatory diseases, in our case stage 3,4 PD might be the cause for the reduction of the lymphocyte count. The same results were obtained in the second part of the

thesis where we confirmed reduction of both absolute and relative numbers of lymphocytes in dogs with stage 3,4 PD when compared to dogs with stage 1 PD. The reduction in absolute numbers of lymphocytes was statistically significant.

As neutrophils are cells that provide first-line defence against infections (Kumar & Sharma, 2010), defects in their number were expected in patients with stage 3,4 PD (moderate to advanced periodontitis) as already demonstrated in human patients with chronic forms of periodontitis (Bhansali et al., 2013; Srinivasan, 2013) but all canine patients with PD presented here and diagnosed with stage 3,4 PD (moderate to advanced periodontitis) exhibited no significant differences in neutrophil count in comparison with healthy controls. As expected, significantly increased neutrophil count was found in a group of dogs with NHNC when compared with healthy controls or with dogs with stage 3,4 PD (moderate to advanced periodontitis). This correlates with literature data suggesting that all canine and human cancer patients may exhibit higher levels of peripheral blood neutrophils, which are involved in the inflammation and are playing an important role in tumour development and progression as already proved by several studies (De Larco et al., 2004; DeNardo et al., 2010; Lee et al., 2012).

Although the values of lymphocytes and neutrophils are in normal ranges the ratio between those cells might result in high N/L ratio and the same was demonstrated in the patients presented here. Decreases in LYM and increases in NEU counts resulted in high N/L ratio in both groups of dogs with stage 3,4 PD (moderate to advanced periodontitis) and NHNC when compared with N/L ratio of healthy controls, despite the fact that patients had the NEU and LYM counts still in normal reference ranges. The results of our study confirmed that the systemic inflammatory response driven by host inflammatory cells is present in both groups of dogs but is more pronounced in dogs with NHNC than in dogs with stage 3,4 PD (moderate to advanced periodontitis). To further support this finding, univariate analysis in patients with stage 3,4 PD (moderate to advanced periodontitis) revealed that N/L ratio was increased in comparison with healthy controls but polytomous logistic regression analysis did not prove significant differences between healthy dogs and dogs with

stage 3,4 PD (moderate to advanced periodontitis) indicating that PD exhibits no significant effect on the systemic inflammatory response, assessed by this biomarker, that would change the number of circulating lymphocytes and neutrophils considerably. As both univariate and multivariate statistical analysis showed significantly higher N/L ratio to be associated with more aggressive processes in dogs (in our case malignant head and neck tumours) and not stage 3,4 PD (moderate to advanced periodontitis), we consider N/L ratio as one of the potential early supportive diagnostic biomarkers, which is able to distinguish between inflammatory, more specifically periodontal disease, and neoplastic conditions affecting dog′s head and neck region.

Further, the differences between N/L ratios among different neoplastic head and neck tumour types indicated that dogs with malignant head and neck tumours (carcinomas, melanomas and sarcomas) exhibited the highest N/L ratios while the lowest N/L ratios were detected in dogs with benign lesions, as expected, with the values close to the N/L ratios of dogs with stage 3,4 PD (moderate to advanced periodontitis). Lower N/L ratio of dogs with benign oral lesions indicates that the inflammatory component of these tumour types is less developed as in malignant head and neck conditions and the finding might support the explanation why these tumours behave less aggressively and do not metastasize. Despite this fact, further studies are needed to reliably suggest the use of N/L ratio as a potential distinguishing biomarker between malignant and benign head and neck lesions in dogs.

Significant increases in the number of PLT in patients with NHNC in comparison with healthy controls correlate with published data suggesting involvement of platelets in the pathobiology of tumours (Bambace and Holmes, 2011; Egan and Hannsidal, 2011; Kono et al., 2012; Shao et al., 2011; Sharma et al., 2014). Increase in PLT count is associated also with other conditions like acute and chronic infections (Klinger and Jelkmann, 2002). This was confirmed in the study presented as patients with stage 3,4 PD (moderate to advanced periodontitis) exhibited significantly higher PLT count in comparison with healthy controls. Despite

significant differences in platelet counts, the values were still in normal ranges in all three groups investigated and were not considered as clinically relevant.

P/L ratios revealed to be significantly different between groups investigated, using univariate and multivariate analysis, showing the highest ratios in patients with NHNC. As for N/L ratio, P/L ratios were increasing with the severity of the disease. In contrast to N/L ratios, significant changes in P/L ratios using multivariate analysis were detected between all groups investigated, also between healthy dogs and dogs with stage 3,4 PD (moderate to advanced periodontitis), where N/L ratio was not significantly altered. This finding indicates P/L ratio being more precise biomarker in detecting the presence of systemic inflammatory response due to different pathological processes affecting head and neck in comparison with N/L ratio that appeared to be more specific for neoplastic head and neck lesions.

Platelet volume has been considered as an indicator of platelet activation (De Luca et al., 2010). More precise estimation of platelet function can be obtained by another CBC biomarker, platelet large cell ratio (PLCR) (De Luca et al., 2010; Seretis et al., 2012). PLCR is determining the number of platelets (expressed in %) with larger volume that is physiologically normal for platelets (De Luca et al., 2010). Increases in PLCR, indicating the effect of tumour cells on the release of platelets from the bone marrow (De Luca et al., 2010; Seretis et al., 2012) did not prove to be significantly different between groups investigated. When adjusted to PLCRi, that is considered even more accurate in estimation of its function, significant differences were detected among groups of dogs investigated with the highest PLCRi detected in patients with NHNC according to the univariate statistical analysis. Using multivariate analysis, PLCRi proved to be significantly different when comparing patients with NHNC and healthy controls or patients with stage 3,4 PD (moderate to advanced periodontitis) and healthy controls. As no difference for PLCRi between healthy dogs and dogs with stage 3,4 PD (moderate to advanced periodontitis) was found this indicates PLCRi, like N/L ratio, to be more specific biomarker for the assessment of the inflammatory response in neoplastic and not inflammatory head and neck conditions, in our case PD. Furthermore, univariate statistical analysis

revealed differences in PLCRi values between different head and neck tumour types in dogs but the results were variable, making conclusions about specificity of PLCRi for specific type of head and neck tumours difficult. Anyway, the highest values of PLCRi were detected in tumours with more aggressive biological behaviour (melanoma and carcinoma) in comparison with sarcomas or epulides but further studies are in progress to assess the role of PLCRi in different head and neck histotypes. An interesting observation was that malignant melanoma cases exhibited the highest values of P/L ratios and PLCRi indicating increased involvement of platelets in the biology of these tumours in comparison with other tumours investigated. High P/L ratio and PLCRi indicate a high correlation between PLT, LYM and tumour cells which might be one of the potential explanations for rapid tumour progression and more frequent metastatic spread of malignant melanoma tumour cells in comparison with other head and neck tumours. The results are in accordance with studies suggesting the contribution of platelets to cancer growth and dissemination (Bambace and Holmes, 2011; De Luca et al., 2010; Seretis et al., 2012). Platelet activation assessed on the basis of high P/L and/or PLCRi indices might help to extract cases as potential targets for the use of drugs such as, for instance, platelet inhibitors, in order to inhibit the progression of tumours and/or spread of tumour cells causing development of metastases.

As the values of MPV/PLT ratios in diseased groups of dogs were similar, no significant differences in MPV/PLT ratio between patients with stage 3,4 PD (moderate to advanced periodontitis) and patients with NHNC were detected. The significant differences in MPV/PLT ratios were found when comparing those two groups of dogs with healthy controls. Therefore MPV/PLT biomarker is not suggested as a potential distinguishing biomarker between inflammatory and neoplastic head and neck conditions as it, according to the results obtained, indicates only the ongoing inflammatory response due to PD or neoplastic processes affecting the head and neck region.

The systemic effects of NHNC and PD on other CBC parameters like white blood cells, eosinophils and red blood cell counts were also detected. A significantly higher

WBC counts were detected in dogs with head and neck neoplasia compared to healthy controls and dogs with stage 3,4 PD (moderate to advanced periodontitis), which is in agreement with studies reporting the presence of leukocytosis in all solid tumour types including tumours of head and neck (Itoh et al., 2009; McKee, 1985; Sakka et al., 2006; Schniewind et al., 2005; Watabe et al., 2011). Despite the fact, that inflammatory mediators in PD cause a systemic inflammatory response indicated by increased number of leukocytes (Al-Rasheed, 2012), we proved the opposite, as no significant differences on WBC count in dogs with stage 3,4 PD (moderate to advanced periodontitis) were detected, again revealing no considerable systemic effect of PD. The same was also found in the second part of the thesis as no significant differences in WBC counts comparing dogs with stage 1 PD and dogs with stage 3,4 PD (moderate to advanced periodontitis) were detected and where the values of WBC were within normal reference ranges in both groups of dogs investigated.

The significant increases in eosinophil counts in dogs with stage 3,4 PD (moderate to advanced periodontitis) and NHNC, when compared to healthy controls, is in agreement with studies indicating many tumour types, most commonly solid epithelial tumours, are associated with tumour and/or peripheral blood eosinophilia (Tefferi et al., 2005; Samoszuk, 1997). The chronicity of PD was found to be the cause for eosinophilia seen in canine patients with stage 3,4 PD (moderate to advanced periodontitis) but was not considered as clinically relevant.

Various studies have tried to evaluate the relationship between PD and anaemia but present conflicting results. Data from human medicine have shown that red blood cell counts, haematocrit and haemoglobin levels are reduced in patients with chronic periodontitis suggesting that chronic stages of PD might be the cause for the development of anaemia (Hutter et al., 2001; Thomas et al., 2006) and improvements in parameters have been detected after periodontal treatment (Agarwal et al., 2009; Rai and Kharb, 2008). Although all patients here were presented with a long lasting disease process e.g. chronic stage of the PD, we did not find any significant differences in these parameters when comparing dogs with stage 3,4 PD (moderate to

advanced periodontitis) and healthy controls which is in accordance with several human studies that failed to show any association between red blood cell parameters and periodontal status (Aljohani, 2010; Havemose-Poulsen et al., 2006; Wakai et al., 1999). Other parameters, like MCV, MCH, MCHC values were not significantly different in dogs with stage 3,4 PD (moderate to advanced periodontitis) when compared to healthy controls and the values were within the normal ranges. Significantly lower values of haemoglobin, haematocrit, MCV and MHC were detected in a group of dogs with NHNC, when compared to healthy controls, and could be ascribed to the presence of paraneoplastic syndrome, as other potential causes for reduced values were not detected.

The overall survival, as expected, was significantly longer in the group of dogs with benign lesions when compared with groups of dogs with malignant head and neck conditions. We specifically mention this finding as there were significant differences in the N/L ratios between groups investigated with lower values of N/L ratios found in a group of dogs with benign and higher N/L ratios in dogs with malignant head and neck conditions. Although the overall survivals were observed to be poorer in dogs with malignant head and neck tumours with high N/L ratios in comparison with patients exhibiting low N/L ratios the difference was not statistically significant. Although the linear increases of pre-treatment N/L, P/L, MPV/PLT ratios and PLCRi have been shown to be associated with worse prognosis and shorter overall survival in humans with tumours with more aggressive biological behaviour this was not confirmed in the study presented (Guthrie et al., 2013; Proctor et al., 2011; Proctor et al., 2013). Our results are in agreement with a study of Mutz et al. (2013) who failed to demonstrate the prognostic impact of N/L ratio in dogs with multicentric lymphoma.

As we were not able to confirm the prognostic value of the CBC biomarkers in dogs with malignant head and neck conditions this might be related to the fact that group of patients was too small and consisted of dogs with different types of head and neck tumours with different biological behaviour. Another important fact that needs to be taken in the account is that the majority of patients with malignant head and neck

conditions were diagnosed at an advanced stage and received different treatments. The treatment with different protocols with palliative or curative intent, which is always a choice of the owner, impacts the overall survival and could have an impact on statistical results as well.

The results of another study performed by the thesis author (Rejec et al., 2015) investigating the impact of CBC indices on overall survival of 150 dogs with different types of malignant tumours and 41 dogs with benign lesions, not only head and neck tumours and all treated with curative intent protocols showed the potential to use the CBC indices as prognostic biomarkers as the survival probability of cancer patients with low N/L, P/L, MPV/PLT and PLCRi was significantly better in comparison with patients exhibiting higher values of CBC indices (data not shown in the thesis) (Rejec et al., 2015).

Therefore, further prospective studies investigating CBC indices in different types of both early-stage and advanced stage malignant head and neck tumours also in comparison with other clinicopathological, histopathological and immunohistochemical biomarkers are in progress. In these cases, not only pre-treatment but also post-treatment assessments of CBC indices may serve as cost-effective therapeutic decision-making and prognostic biomarkers.

In the first part of the thesis we have demonstrated the differences in the systemic inflammatory immune responses provoked by both inflammatory and neoplastic head and neck conditions in dogs assessed by CBC parameters and CBC indices with more obvious changes in CBC indices detected in dogs with neoplastic head and neck conditions than in dogs with stage 3,4 PD. Despite that finding we believe, that dysregulations in immunity of patients with PD exists and may be the cause of insufficient or unbalanced immune/inflammatory responses.

Therefore, the immune/inflammatory background of PD may be elucidated through investigation of immune mechanisms that help to maintain a balance between activation and suppression of the immune responses. In these processes, both innate

and adaptive immunity play a role (Silva et al., 2015). A healthy periodontium can be maintained only if the immune responses are able to combat with periodontopathogens on one side while reducing immune-mediated pathology processes and (auto)-immune reactions on the other side. Regulatory mechanisms of these processes are mediated also by Tregs, a focus of the investigation in the second part of the thesis, which suppress the activation, proliferation and effector function of a variety of immune cells not only in neoplastic but also inflammatory diseases (Belkaid and Tarbell, 2009).

Changed frequencies of Tregs have been already demonstrated in several inflammatory diseases, also in PD (Garlet et al., 2010). Several authors demonstrated that Tregs, despite having a role in protecting the host from development of the excessive immune-mediated pathologies, have also the ability to inhibit anti-microbial effector responses thus helping the pathogens to survive and maintain the infection (Belkaid and Rouse, 2005; Boer et al., 2015; Majlessi et al., 2008; Mills, 2004; Robertson and Hasenkrug, 2006).

Specifically in terms of PD, the role of T cells in mediating the immune responses is well-recognized and the balance between different sets and subsets of T cells, investigated also in the study presented, has been shown to be of the main importance in the immunoregulatory control of PD (Cardoso et al., 2008; Garlet et al., 2010; Gemmell et al., 2001; Nakajima et al., 2005; Takeuchi et al., 1991; Yamazaki et al., 1995) although the exact role and mechanisms still need to be clarified. Dogs with naturally occurring PD serve as a very good model for the investigation of PD in both dogs and humans (Hoffmann and Gaengler, 1996; Nemec et al., 2013) as experimentally induced PD might lack the components of the immune system participating in the initiation of this prevalent inflammatory/infectious disease. If the role of Tregs in PD is confirmed, the manipulation of the Treg cell number and/or function can help to develop new treatment strategies in order to prevent the development and/or progression of one the most common infectious diseases, in both humans and dogs, which at the end stage unfortunately always leads to tooth/teeth loss.

Although a wide range of pathogens induces Tregs, distinct effects of Tregs have been demonstrated for different pathogens and in different stages of acute *vs* chronic viral or bacterial infections (Boer et al., 2015). Although the suppressive role of Tregs, accumulating in the gingival tissues during PD in human and animal experimental models, has been shown to modulate the local immune responses (Cardoso et al., 2008; Dutzan et al., 2009; Garlet et al., 2010; Nakajima et al., 2005; Okui et al., 2008; Takeuchi et al., 1991) the data regarding the systemic levels of Tregs in patients with PD are lacking (Cardoso et al., 2008; Indumathy et al., 2012). As PD may also result from the failure of peripheral tolerance mechanisms, including Tregs, it was of our interest to investigate whether stage 3,4 PD (moderate to advanced periodontitis) in dogs correlates with altered Treg cell homoeostasis systemically.

Thymus-derived natural $CD4^+CD25^+$ Tregs were investigated, as they are suppressing the immune response in several inflammatory and autoimmune diseases, and PD might be one of them (Baecher-Allan et al., 2001; Foussat et al., 2003; Valencia et al., 2007). These suppressor cells neutralize other immune cells by various mechanisms suppressing undesired or even harmful immunological reactions to self or foreign antigens (Piccirillo et al., 2004; Read et al., 2000; Shevach, 2002). Cell surface biomarker CD25 and intracellular biomarker FOXP3, accepted as reliable phenotype markers of Tregs, not only in humans but also in dogs, were used (Pinheiro et al., 2011). Several authors demonstrated the expression of FOXP3 in $CD4^+CD25^+$ T cells, especially in the $CD4^+CD25^{bright}$ cells ($CD25^{bright}$ cells; cells expressing high levels of CD25) with regulatory functions (Frey and Brauer, 2006; Lin et al., 2007; Mottet et al., 2003). Only $CD4^+$ cells with high expression of CD25 have efficiently suppres the proliferative responses, thus being considered as true Tregs (Baecher-Allan et al., 2001). In the study presented $CD4^+CD25^+$ T cells investigated comprised a mixed population of $CD4^+$ T cells expressing low-intermediate-high percentage of CD25. We found that not all, but approximately only one-third of $CD4^+CD25^+$ T cells detected expressed FOXP3. Pinheiro et al. (2011) demonstrated that 5% of $CD4^+$ T cells showing the highest CD25 expression ($CD4^+CD25^{high}$) were enriched in cells expressing FOXP3. The frequency of

$CD4^+CD25^+$ T cells expressing FOXP3 in our study is similar to the percentage of $CD4^+CD25^{high}FOXP3^{high}$ T cells detected in a study of Pinheiro et al. (2011). According to this finding, we can speculate that $CD4^+CD25^+$ T cells expressing FOXP3 in the study presented are $CD4^+$ T cells with high levels of CD25 ($CD4^+CD25^{bright}$ T cells) and can be thus considered as true Tregs (Baecher-Allan et al., 2001; Pinheiro et al., 2011).

Significantly lower peripheral blood and regional lymph node levels of Tregs in dogs with stage 3,4 PD (moderate to advanced periodontitis) in comparison with dogs with stage 1 PD (gingivitis) are indicative of an impaired immunity and are most potentially a consequence of a significant reduction in $CD4^+$ T cells, confirmed also in dogs with stage 3,4 PD (moderate to advanced periodontitis). Further, a significant decrease of $CD4^+CD25^+FOXP3^+$ Tregs detected in dogs with stage 3,4 PD (moderate to advanced periodontitis) indicates on an impaired suppressive function of these cells as this phenotype indicates their suppressive potential (Ziegler et al., 2006). The same was found by Pinheiro et al. (2011), who showed, that peripheral blood $CD4^+CD25^{high}FOXP3^{high}$ T cells in dogs show regulatory phenotype and suppressive function *in vitro*.

Our results are in agreement with studies in both human and animal models, which have demonstrated that a decrease of Tregs in peripheral blood contributes to immune disorder related diseases and PD could be one of them. If a decrease in the number of Tregs in the study presented is a cause or a consequence of stage 3,4 PD (moderate to advanced periodontitis) remains to be elucidated as the patients were not followed in a longer period of time, more precisely before the onset of the development of more advanced stages of PD where attachment loss is present for instance stage PD2 and further stage 3,4 PD (moderate to advanced periodontitis) and after treatment. An interesting finding in the study was the identification of some patients with stage 1 PD (gingivitis) and patients with stage 3,4 PD (moderate to advanced periodontitis) exhibiting similar values of Tregs. Analysis of Tregs in such cases might serve as a useful diagnostic/prognostic tool to identify and follow individuals with lower levels of Tregs who might be at risk of developing more

serious stages of PD in the future.

Although the pathogenesis and progression of PD is immune-mediated it is also genetically dependent (Moutsopoulos and Madianos, 2006). Genetic factors, reported to have an important impact on thymic output of Tregs, might be one of the causes for reduced production of Tregs (Buckner, 2010). If lower levels of Tregs in the peripheral circulation and regional lymph nodes in dogs with stage 3,4 PD (moderate to advanced periodontitis) are a consequence of genetic factors needs to be elucidated in further studies.

Next, autoimmune disorders are characterized by the loss of self-tolerance, which can be partly attributed to alterations of regulatory T cell populations. Although PD is not considered primarily as an autoimmune disease, there are reports indicating that PD has an autoimmune component (Anusaksathien et al., 1992; De-Gennaro et al., 2006; Hirsch et al., 1988; Nair et al., 2014; Tabeta et al., 2000; Yamazaki et al., 2002). The significant reduction in the regulatory T cell populations detected in dogs with stage 3,4 PD (moderate to advanced periodontitis) could occur as a result of defects in the thymopoeisis, more precisely defects in T-cell compartment in the thymus that further affect the thymic development of Tregs and led consequently to development of an autoimmune disorder, in our case stage 3,4 PD (moderate to advanced periodontitis) (Nair et al., 2014; Shevach, 2002). The results obtained are in agreement with authors of several studies who indicated that the loss or the reduction of natural Tregs leads to the development of autoimmune disorders. If this is true, the emergence of autoimmunity that could be related to diminished frequency of Tregs in peripheral circulation and regional lymph nodes in dogs with advanced stages of PD, for instance stage 3,4 PD (moderate to advanced periodontitis) can be suggested.

Although Tregs in the study presented were not measured locally, next potential explanation for the reduced peripheral blood and regional lymph nodes frequency might be peripheral migration of Tregs to the site of inflammation like in other immune-mediated diseases, an example are systemic lupus erythematosus and

rheumatoid arthritis in humans (Chavele & Ehrenstein, 2011). As already investigated by several authors, natural Tregs preferentially accumulate at the site of the inflammation, also in PD, where they limit the effector immune responses and promote pathogen survival. In human periodontitis patients several reports have demonstrated increased infiltration of Tregs with the proportion of $CD4^{+}CD25^{+}$ T cells in lymphocytes and FOXP3 extracted from gingival tissues higher than that in peripheral blood (Takeuchi et al., 1991; Okui et al., 2012; Duarte et al., 2011; Nakajima et al., 2005; Kim et al., 2010). In the study presented the local inflammation in gingival tissues was present in all dogs with stage 3,4, PD (moderate to advanced periodontitis) and according to the facts mentioned it could recruit Tregs from the periphery and subsequently lead to the reduction of circulating Tregs in peripheral blood and regional lymph nodes.

Maintenance of peripheral Tregs is a dynamic process, influenced by cytokine microenvironment, which favours the induction of Tregs in the peripheral circulation (Wolf et al., 2005). Systemic effects of PD are related to the permeability of periodontal tissues to inflammatory cells and consequent production and accumulation of of pro-inflammatory cytokines, which might have suppressive function towards Tregs. One of such cytokines is TNF-α found to be associated with the presence of periodontopathogenic bacteria (*A. actinomycetemcomitans* and *P. gingivalis*) (Cray, 2012; Gabay & Kushner, 1999). Peripheral blood TNF-α has been shown to be elevated in rats and humans with PD (Passoja et al., 2010). Although not measured in the study presented, TNF-α might be suppressive to Tregs, reducing their number in peripheral circulation. Several other factors like CD28, TGF-ß, dendritic cells (DC), IL-2, IL-4, IL-7 and IL-15, although not investigated in the study presented and are involved in the pathogenesis of PD, guide proliferation, function and survival of Tregs (Cray, 2012; Gabay & Kushner, 1999). IL-2 is important for the growth of Tregs supporting their development in the thymus and the maintenance of peripheral homeostasis by signalling through CD122. *P. Gingivalis*, one of the most common periodontopathogens in both humans and dogs, can suppress the accumulation of IL-2, which further attenuates T cell proliferation and alters adaptive immune responses (Khalaf & Benthsson, 2012). TGF-ß is one of

the cytokines secreted by Tregs and is associated with survival and function of Tregs promoting their differentiation by regulating the signalling pathways and induction of FOXP3 expression (Peng et al., 2004). TGF-ß has been shown to be associated with periodontal inflammation in both humans and in dogs (Gurkan et al., 2006; Skalerič et al., 1997). The levels of TGF-ß1 in beagle dogs were elevated in moderate PD but with the progression of the disease significant decreases of TGF-ß1 in gingival fluid samples and serum of dogs with experimentally induced advanced periodontitis were demonstrated (Skalerič et al., 1997). We can speculate that insufficient production of TGF-ß in dogs with stage 3,4 PD (moderate to advanced periodontitis) could attribute to reduced frequency and impaired function of Tregs and consequent promotion of the development and persistence of PD in dogs.

"In periodontitis, the effector immune response has to be regulated to control the bacterial growth and dissemination and to prevent tissue damage" (Kalburgi et al., 2013). The number of immune cells and their effector functions are under strict control of Tregs. We additionally confirmed significantly lower values of other peripheral blood lymphocyte subpopulations ($CD4^+$ $CD8^+$ and $CD5^+$ T cell-subsets and $CD21^+$ B cells) in dogs with stage 3,4 PD (moderate to advanced periodontitis) compared to dogs with stage 1 PD (gingivitis), which might be the effect of disturbed Treg cell homoeostasis. If reduced helper and suppressor T cell frequencies existed before the onset of the PD or developed as a consequence of the disease or were determined by individual intrinsic factors remains to be elucidated in further studies.

Helper T cells ($CD4^+$) are activators of humoral and cellular-mediated immunity playing an important role in recognising and fighting against bacterial infections, also PD (Yamazaki et al., 1995). $CD4^+$ T cells have been reported to accumulate in gingival tissues participating in the inflammatory process promoting the severity of the disease (Takeichi et al., 2000; Yamazaki et al., 1995). These cells are also suggested to be responsible for the chronicity of the disease (Jotwani et al., 2001; Takeichi et al., 2000; Yamazaki et al., 1995). In PD, periodontopathogens activate peripheral host's defence mechanisms and stimulate peripheral migration of cells locally (Cardoso et al., 2008) by altering T cell responses (Khalaf & Benghtsson, 2012) This was showed by Yilmaz et al. (2008) who confirmed the association of

$CD4^+$ T cells with *P. gingivalis* and indicated that $CD4^+$ T cells move to the site of inflammation where they modulate cytokine production and protective (Th1) or destructive (Th2) immune responses. Decreases of $CD4^+$ T cells in peripheral blood detected in our study might be ascribed to the migration of these cells to periodontal tissues lowering the values of $CD4^+$ T cells in the periphery. This is in agreement with studies showing a reduced number of $CD4^+$ T cells in peripheral blood of human patients with chronic forms of periodontitis (Erciyas et al., 2006; Orbak et al., 2003). That PD decreases peripheral blood $CD4^+$ T cells was shown also in experimentally ligature-induced PD (Manti et al., 1984).

How cytotoxic T cells ($CD8^+$) that are regulated by Tregs (Cifcibasi et al., 2015) participate in PD is not fully elucidated yet but it is agreed that their role relates to the $CD4^+$ T cell responses (Pfifer et al., 1993). $CD4^+$ T cells and $CD8^+$ T cells regulate each other *in vivo* and defects in one T cell subtype might cause further imbalance of the other subset (Mosmann & Coffman, 1989; Read et al., 2000) with the end result of these uncontrolled activation leading to tissue damage (Artese et al., 2011; Cifcibasi et al. 2015). Stimulative effect of $CD4^+$ T cells to B cells in antibodies production, recruiting and activating inflammatory cells (macrophages, neutrophils, eosinophils and basophils) to the site of inflammation are well described (Mosmann & Coffman, 1989). Inadequate numbers of $CD4^+$ T cells detected in the study presented could be responsible for ineffective B cells responses (as detected by low number of CD21 B cells) and consequently impaired or unsatisfactory defence against periodontopathogens. Potentially ineffective B cell responses can be ascribed to significant reduction of peripheral B cells as detected by low expression of $CD21^+$ B cells in dogs with stage 3,4 PD (moderate to advanced periodontitis) when compared to dogs with stage 1 PD (gingivitis). According to the literature data published, a depression of $CD8^+$ T cells due to PD occurs but is more stable in comparison with $CD4^+$ T cells (Kinane et al., 1989). Fluctuations of $CD4^+$ T cells due to PD exist and reflect the individual disease progression, being depressed during active or progressive phase and returning to normal when periodontal process reverts to stable, inactive state (Katz et al., 1988; Kinane et al., 1989). This might explain the inconsistent reports regarding the percentages of subpopulations of T cells and

CD4/8 ratios. In our study chronic but active stage of stage 3,4 PD (moderate to advanced periodontitis) in dogs attributed to similar depression patterns of both T cell subpopulations investigated ($CD4^+$, $CD8^+$), although $CD8^+$ T cells in the study presented revealed to be depressed more than $CD4^+$ T cells. The results of ratios between peripheral blood $CD4^+$ and $CD8^+$ T subpopulations of human patients with PD are mixed (Katz et al., 1988; Kinane et al., 1989; Negasawa et al., 1995; Takahashi et al., 1995). No alterations in CD4/8 ratios in early and chronic stages of PD were detected but depressed CD4/8 ratios have been confirmed in patients with juvenile PD and rapidly progressive PD (Kinane et al., 1989). In our study CD4/8 ratio was found to be elevated but not statistically significant which is in accordance with studies of Katz et al. (1988) and Orbak et al. (2003) who found no significant differences in CD4/8 ratios between groups of patients investigated.

As the pathogenesis of the PD is very complex (Teles et al., 2013) it is difficult to find the cause-effect relationship responsible for the development of advanced periodontal lesions. The results of the second part of the thesis indicate on the involvement of regulatory T cells and other lymphocyte T and B subpopulations in the periodontal disease pathogenesis that might be responsible for multiplication, activity and persistence of periodontopathogens and could in the further studies represent a target for new treatment strategies of this devastating disease in both dogs and humans.

As proved in the first two parts of the thesis the inflammatory and neoplastic head and neck conditions having its own characteristics exert different systemic effects confirmed by differences in CBC indices in both conditions and regulatory T cells along with other lymphocyte subpopulations in PD. Although both neoplastic and inflammatory head and neck conditions are classified in several ways, including clinical investigation into the extent of the disease (clinical staging), classification of the host and the disease, histologic diagnosis, grading and prognostic significance (Arzi & Verstraete, 2012), many questions regarding the pathogenesis of above mentioned conditions remain unclear and open. New insights into the biology of both inflammatory and neoplastic head and neck conditions at the molecular level are

necessary as they could lead to therapies that may block inflammatory/immune and the carcinogenic processes especially during the early stage and keep local/distant disease under control. A better understanding of the mechanisms behind the aggressiveness of malignant canine head and neck tumours, especially those of tonsillar and sino-nasal origin which are the most difficult to treat, is necessary. Differences in the biologic behaviour as well in the prevalence of local recurrences and/or distant metastatic disease, occurring frequently in these types of cancers, may develop as a consequence of abnormalities in intracellular signalling pathways where both proliferation of tumour cells and angiogenesis play an important role (Farber et al., 1984; Ferrara, 1999; Hicklin & Ellis, 2005). Our next aim was, therefore, to investigate the differences between inflammatory, benign and malignant head and neck conditions in dogs that might be related to the different expression pattern of the angiogenic and proliferative factors, like VEGFR-2 and Ki-67.

The VEGF, one of the most important mediators of angiogenesis, is normally in low levels expressed in both human and animal tissues (Folkman, 1990; Papetti & Herman, 2002; Ziche & Gullino, 1982). High intratumoral VEGF expression correlates with VEGF function through its three receptors (VEGFR-1, -2, -3) acting on endothelial cells of existing blood vessels and promoting new blood vessel formation (Ferrara et al., 2003). VEGFR-2 was chosen as several evidence suggest the involvement of VEGFR-2 in the angiogenesis of the neoplastic processes (Ferrara et al., 2003; Ferrara, 2004; Kim et al., 2006; Olsson et al., 2006;).

The role of VEGFRs has been confirmed in the pathogenesis of several diseases of inflammatory, (auto)-immune-mediated origin such as inflammatory bowel disease or rheumatoid arthritis for instance (Costa et al., 2007; Detmar et al., 1994; Scaldaferri et al., 2009) and neoplastic diseases (Waldner et al., 2010). Data regarding the expression of VEGFR-2 in benign and inflammatory oral conditions are lacking in both human and veterinary medicine. Although no immunohistochemical expression of VEGFR-2 in epithelial cells of oral inflammatory lesions and neoplastic cells of oral benign tumours was confirmed in the study presented, it needs to be mentioned that VEGF and its receptors might have

been expressed as well in these conditions. Our results are similar to the results of human studies indicating normal and mildly dysplastic oral epithelium expresses no VEGF/VEGFRs or expresses significantly lower values, respectively, when compared to neoplastic samples (Eisma et al., 1997; Denhart et al., 1997; Li et al., 2005).

In the context of inflammatory and neoplastic diseases, it needs to be mentioned that inflammation as such could contribute to the development and progression of various cancers (Angelo & Kurzrock, 2007). VEGF signalling in angiogenesis might, therefore, represent an important correlation between inflammation and tumour development but the molecular mechanisms of angiogenesis in inflammation associated with cancer development are still poorly understood and remain to be elucidated (Waldner et al., 2010).

In the study presented we demonstrated the differences in clinicopathological and immunohistochemical features of malignant tumours arising from the head and neck with special regard to VEGFR-2 expression and confirmed its potential role in oncogenesis and prognosis of more advanced malignant tumours of the head and neck in dogs. The molecular expression of VEGFR-2 in malignant but not benign and inflammatory head and neck conditions was confirmed in 53.3% of tumour cells of epithelial and 64.7% of other tumours consisting of MM+SAR head and neck tumours. Data obtained are in agreement with studies in human medicine confirming the expression of VEGFRs in tumour cells of several human neoplastic tissues of urogenital, pancreatic, mammary, colorectal or non-small lung cell carcinomas including head and neck carcinomas (Amaya et al., 1997; Donnem et al., 2010; Gratzinger et al., 2009; Ryden et al., 2003; Seto et al., 2006). In veterinary medicine VEGFR-2 protein has already been detected in several types of canine carcinoma and sarcoma tumours (oral and cutaneous melanomas, mammary tumours, cutaneous SCC and trichoepitheliomas, hemangiosarcoma, cutaneous fibrosarcoma, apocrine gland anal sac and thyroid carcinoma) (Al-Dissi et al., 2007; Al-Dissi et al., 2009; Al-Dissi et al., 2010; Millanta et al., 2006; Rawlings et al., 2003, Rebuzzi et al., 2007; Restucci et al., 2004; Urie et al., 2012, Yonemaru et al., 2006). Flickinger et al.

(2013) demonstrated the cytoplasmatic VEGFR-2 in canine oral melanoma cell line and reduction in the activity of VEGFR-2 after radiation with increasing dosages.

Our results agree with the human data where positive staining of VEGFR-2 on tumour cells of head and neck SCC was demonstrated (Kyzas et al., 2005; Lalla et al., 2003). We noted positive immunohistochemical reaction for VEGFR-2 in tumour cells of malignant head and neck conditions with cytoplasmic expression. In canine head and neck tumours with high expression of VEGFR-2, a higher expression of the receptor in the centre of the tumour nests when compared with those on the periphery. We immunohistochemically, for the first time, demonstrated VEGFR-2 expression in canine sino-nasal SCC which expressed the highest percentage of VEGFR-2 in comparison with all other malignant head and neck tumours. The observation in these tumours types can be explained by the presence of an adaptive response of the tumour cells to lower perfusion and consequently to a relatively lower oxygenation (that develops as a consequence of hypoxia, a frequent finding in tumours) which facilitates the increased expression of VEGF/VEGFRs promoting growth and development of new vessels providing enough oxygen for new tumour cell proliferation (Papetti & Herman, 2002; Stinga et al., 2011).

Additionally, we observed that VEGFR-2 overexpression moderately correlated with the presence of tumour necrosis. This is in accordance with several studies demonstrating that VEGF expression is attenuated in tumour cells close to areas of necrosis (Brown et al*.,* 1993). According to Du et al. (2003), this may be explained by hypoxia, which stimulates VEGF expression and its biological activity. In addition, Leek et al. (1999) suggest that an association between higher vascular density and increasing necrosis exists.

According to the expression pattern of VEGFR-2 demonstrated in canine malignant head and neck tumours, VEGFR-2 may serve as one of the potential regulators of tumour aggressive behaviour having a significant role in carcinogenesis by mediating tumour angiogenesis, especially in canine sino-nasal SCC. Furthermore, the differences in the expression pattern of VEGFR-2 between benign+inflammatory *vs* malignant or oral *vs* sino-nasal head and neck tumours, confirmed in the study

presented, might offer a potential explanation for the differences in their less or more aggressive biological behaviour.

Further investigation was focused on the detection of Ki-67, an important prognostic biomarker in human and also veterinary oncology, which labels cells in the proliferative phase (S, G2, M and G1) and indicates the cell proliferative activity (Yu et al., 1992). Ki-67 offers a rapid and reliable estimate of the neoplastic cell growth fractions (Gerdes et al., 1986). We detected the expression of Ki-67 in all cases investigated with the lowest expression of Ki-67 in inflammatory and benign oral conditions when compared with malignant ones. Among malignant cases epithelial head and neck tumours expressed more Ki-67 and the intensity of expression of Ki-67 in comparison with MM+SAR head and neck tumours was stronger. An interesting finding was that inflammatory sample tissues expressed more Ki-67 in comparison with benign sample tissues where the intensity of expression appeared negative or was very mild. The results of the study also showed that Ki-67 was significantly higher in tumour samples with strong reactivities to VEGFR-2 than those with weak reactivities. We confirmed that all sino-nasal SCC tumours investigated, which were demonstrated to express the highest level of VEGFR-2, had the highest proliferative activity. The correlation between proliferation and angiogenesis of tumour cells, the major source of VEGF and the increase of proliferative index is ascribed to the angiogenic activity of VEGF, which can also be true for this group of tumours (Takahashi et al., 1995). The hypothesis is that VEGFs form an autocrine loop with VEGFRs (VEGFR-1 and VEGFR-2) expressed on tumour cells thereby enabling a tumour to regulate its own survival, growth and progression (Fan et al., 2005; Tanno et al., 2004). High proliferative activity of tumour cells in sino-nasal SCC may, therefore, be potentially induced by the presence of hypoxia in these tumour types stimulating angiogenesis (Semenza & Wang, 1992; Shweiki et al., 1992). During tumour growth the distance between tumour cells and blood vessels increases which negatively affects the delivery of oxygen and nutrients (Waldner et al., 2010). The tumour cells adapt to this hypooxygenated environment by stimulating new angiogenesis (Waldner et al.,

2010). Well-vascularized and oxygenated environment then allows the tumour to grow as tumour cells can proliferate further (Ferrara, 2009).

Shiomitsu et al. (2009) demonstrated the expression of VEGF in canine epithelial tumours of the nose. Of 24 tumour samples 22 (91.7%) stained positive for VEGF. In accordance with the results of his study, significantly higher expression of VEGFR-2 and Ki-67 in canine sino-nasal epithelial tumours by our study could support the hypothesis that VEGF may be an autocrine growth factor for epithelial tumour cells and that the autocrine growth regulatory role may be mediated through VEGFR-2. Furthermore, over-expression of VEGFR-2 and Ki-67 in sino-nasal tumours may thus correlate with more aggressive tumour behaviour, advanced stages and poor prognosis and this was true for all canine patients investigated. All dogs with sino-nasal SCC expressing higher levels of VEGFR-2 and Ki-67 were diagnosed at an advanced stage and the prognosis in comparison with other dogs investigated was worse. The finding might be supported by the presence of tumour hypoxia on one side and high proliferative activity of tumour cells on the other side. These conditions have been demonstrated to be associated with poor therapeutic outcome (Harris, 2002; Zhang et al., 2003). Low proliferative activity of tumours arising from the head and neck correlated with poor treatment outcome in comparison with tumours that had higher proliferative activity (Couture et al., 2002; Freudelsperger et al., 2012; Kennedy et al., 1997). The rapid response to radiotherapy of highly proliferative tumours demonstrated in the study presented in 4/7 dogs with advanced head and neck SCC is confirming this finding. It is true that high proliferative activity of tumours leads to rapid responses to radiotherapy but it needs to be investigated further if the high expression of these two biomarkers might be also responsible for more rapid recurrences of these tumours. An additional therapeutic benefit in these cases could be achieved by targeting the VEGFR-2 pathways and/or other proliferative and hypoxia pathways involved in the pathogenesis of these tumour types.

Although not investigated in the study presented assessment of intratumoural microvessel density (MVD), which is considered to be a marker of the neo-angiogenesis process as it reflects the amount of small blood vessels within the

tumour (Orre & Rogers, 1999) could offer additional information. VEGF/VEGFRs expression correlated with high MVD and poor prognosis in human cancer patients (Takahashi et al., 1995; Toi et al., 1995; Yamamoto, Konishi et al., 1997). It has been demonstrated that MVD determined in primary tumours is significantly associated with local growth, metastases and prognosis in for instance human breast, prostate carcinomas and haematological malignancies and is most predictive in those tumours that induce significant angiogenesis (Nico et al., 2008). Canine seminomas (Restucci et al., 2003), SCC (Al-Dissi et al., 2007) or lymphomas (Wolfsberger et al., 2008) showed increased MVD as well. The data regarding the value of MVD in the context of the radiotherapy treatment of advanced tumours of the head and neck is still debatable, as some authors proved that higher MVD in tumour biopsy specimens significantly correlated with better radioresponse while others proved the opposite (Zhang et al., 2003). Since angiogenesis is induced by hypoxia, neovasculature should be taken as an indicator of hypoxia according to some authors (Aebershold et al., 2000; Zhang et al., 2003).

Different results were obtained for canine oral SCC. Despite the expression of all three receptors (VEGFR-1, 2, 3) on the tumour and vascular endothelial cells (the main source of VEGFs/VEGFRs) of human oral SCC, the VEGFR-2 is most common receptor detected (Kyzas et al., 2005; Lalla et al., 2003; Moriyama et al., 1997; Neuchrist et al., 2001). In contrast with sino-nasal SCC, only 20% of oral SCC cases expressed VEGFR-2 and the percentage of the expression was very low. According to IHC scoring, these tumours were considered as VEGFR-2 negative. The results obtained in the study presented correlate with the results of several human studies indicating no positivity for VEGFR-2 in HNSCC then studies revealing positivity for VEGFR-2 detected in those tumour types (Neuchrist et al., 2001; Sato & Takeda, 2009). Furthermore, a significant difference between oral carcinoma tumours, when compared with oral malignant melanoma tumours, was detected with the latter expressing more VEGFR-2. Similar to the results of Rawlings et al. (2003) who previously confirmed VEGFR-2 positivity in both oral and cutaneous melanoma samples in dogs, all oral malignant melanoma samples studied, stained positive for VEGFR-2, although the expression varied from 1-30%.

Higher expression of VEGFR-2 in canine oral MM and SAR tumours in comparison with oral epithelial tumours may indicate on their different biological behaviour, also in the context of development of distant metastases which were present in 18% (3/17) of dogs with MM+SAR tumours (1 osteosarcoma and 1 rhabdomyosarcoma). An interesting observation was that the sample tissues of those patients showed the strongest expression of VEGFR-2 among all mesenchymal samples. On the contrary, the expression of VEGFR-2 in the rest of 71% (12/17) mesenchymal tumour samples was low or negative and all the patients were free of metastasis. Only two patients from this group after treatment developed and died due to pulmonary metastasis, both of them were malignant melanoma cases while the reason for death of other patients was local tumour progression or other systemic diseases. High expression of VEGFR-2 in a study of Simiantonaki et al. (2007) has been shown to have an inhibitory effect on development of distant metastasis in human patients with colorectal carcinoma. The results of sino-nasal carcinoma cases, expressing high percentage of VEGFR-2, are supportive of this finding, as none of the patients was diagnosed or developed or died due to distant metastatic disease despite advanced tumour stages. How and if high expression of VEGFR-2 in canine patients with MM and SAR oral tumours affects the development of distant metastasis, needs to be clarified in further studies, as the number of cases in the study presented is too low that would allow making any relevant conclusions regarding this as tumoral expression of not only VEGFR-2 but also VEGFR-1 may have both protective effect or can act as negative regulator for processes facilitating metastases.

Ki-67 and VEGFR-2 expression in neoplastic cells of canine head and neck tumours investigated showed no significant correlation with degree of differentiation and grade of the tumours. Despite this finding, there was a trend towards positive relation between the VEGFR-2 expression and the degree of histological differentiation. As in the study of Stinga et al. (2011) poorly and moderately differentiated tumours expressed less VEGFR-2 while the majority of tumours expressing the highest level of VEGFR-2 were well-differentiated tumours.

The most important factors, which dictate the potential overall effectiveness of the treatment modalities chosen, and of course, have an impact on the survival of HN

tumour patients are, site, size, stage and histopathological biomarkers of the tumours (Bergman & Wolchok, 2008). Although histopathological variables investigated including histological type, grade, mitotic rate, invasiveness and/or degree of differentiation, differed between different head and neck histotypes, the variables did not prove as predictors of survival of dogs with neoplastic head and neck tumours investigated. There was a trend of increased overall survival of dogs with low number of mitosis and dogs with well-differentiated tumours, but the difference was not statistically significant. In contrast, site (location), stage, grade and the presence of necrosis affected the overall survival of dogs investigated. Dogs with oral tumours lived significantly longer than dogs with sino-nasal tumours. Advanced stages of the head and neck tumours and the identification of necrosis in the pathohistological specimens correlated with shorter overall survival. Furthermore, the negative prognostic significance of VEGFR-2 in dogs with neoplastic head and neck conditions was confirmed. Our results are in agreement with studies indicating the overexpression of VEGFs/VEGFRs is associated with tumour aggressiveness, poor treatment outcome and decreased survival in both humans and companion animals. The results of several studies from human and also veterinary medicine demonstrated high positive rates of Ki-67 as unfavourable prognostic factor (Bergkvist et al., 2011; Nichols et al., 2012; Scase et al., 2006; Sittel et al., 1999). In contrast with VEGFR-2, the proliferative marker Ki-67 did not prove as negative prognostic factor in dogs with malignant tumours of the head and neck despite observation that low expression of Ki-67 was associated with longer overall survival. The results agree with the results of the study of Fu et al. (2014) who confirmed no significant relationship with survival time despite the tendency for high proliferative index to be correlated with poor survival of dogs with nasal carcinoma.

Overexpression of VEGFR-2 in advanced canine head and neck tumours was significantly associated with poorer response to treatment and shorter overall survival times. As the role of VEGFR-2 as unfavourable prognostic biomarker in dogs with malignant head and neck conditions was confirmed further work is focused to ascertain how different expression levels of VEGFR-2 can be used in screening, diagnosis, monitoring and prognosis of dogs with different types of head

and neck neoplasia. Furthermore, dogs with head and neck tumours of malignant origin expressing VEGFR-2 may benefit from using the biologically target drugs inhibiting angiogenesis as monotherapy or as a part of multimodal treatment approach.

Disease control and organ function preservation are two the most important goals of the treatment of advanced SCCs affecting head and neck in both human and veterinary medicine with radiotherapy as one of the crucial and most common treatment modalities applied. Improvements in the treatment strategies are needed as they can improve the overall treatment response and prognosis of patients with these types of cancer. No generally accepted consensus regarding treatment strategy/strategies in both human and veterinary medicine indicates that treatment of these types of tumours is difficult and very often unpredictable. Diversities in gene and/or protein expression in tumours of head and neck might also explain response variations of the patients to the same treatment modality and immunohistochemical analysis of those tumours might help to improve the treatment strategy. Furthermore, treatment decision making for these types of cancer must take into the consideration not only the optimal treatment strategy for local/regional tumour control but also associated toxicities and potential morbidity due to treatment modalities chosen.

The population of canine patients included in the last part of the thesis were patients with advanced SCCs of the head and neck. All dogs were otherwise healthy but advanced stages of head and neck SCC made these cases poor surgical candidates. Instead, radiotherapy appeared as only treatment modality possible. Radiation treatment possibilities for canine cases presented here included the use of a standard definitive daily radiation therapy protocol consisting of 3 Gy per fraction in 16-18 treatment days to a total dose of 48-54 Gy (Liptak & Withrow, 2007). Although an accelerated chemoradiotherapy protocol consisting of 3.5 Gy fractions to a total of 49 Gy delivered twice daily in 9 days described by Fidel et al. (2011) has not been described for the treatment of advanced canine head and neck SCC, it was suggested as an alternative option in the canine cases presented. The rationale was the possible specific biological behaviour of these tumours, especially the aggressive tonsillar SCC and sino-nasal SCC cases, the short overall treatment and hospitalisation times

(standard and high dose definitive protocol 22-24 days *vs*. only 9 days) and lower treatment costs (less treatment fractions and anaesthetics, less time in hospital) which were factors that convinced clients to choose the latter. Other reported accelerated regimen using a larger dose per fraction (e.g. accelerated hypofractionated radiation protocol; 4.8 Gy daily over a period of 10 days to a total dose of radiation of 48 Gy) could also be used, but this could potentially lead to an increased risk of delayed toxicity due to higher dose fraction used.

Accelerated repopulation of tumour cells during the conventional fractionated radiotherapy has been recognised as an important determinant of local control and possible cause of treatment failure in human head and neck squamous cell carcinomas, especially if the overall treatment time is prolonged (Trott & Kummermehr, 1985). This hypothesis is supported by laboratory as well as clinical data, which showed median potential doubling times in many SCC tumours of only approximately 4 days (Bentzen, 2003; Withers, 1985). These observations have led to the development of alternations in fractionation protocols, whereby radiotherapy is administered in a shorter overall time using multiple daily fractions. In accelerated fractionation protocols the overall treatment time is reduced while the fraction size and total dose remain unchanged (Harai et al., 2005). The reduction in the overall treatment time prohibits the regeneration and consequent repopulation of tumour cells (Harari et al., 2005). On the other side, the reduction in overall treatment time however influences the response of healthy tissue and will lead to an increase in acute side effects (Fu et al., 2000; Harari et al., 2005; Horiot et al., 1997).

Theoretical differences between protocols can be evaluated by comparing the biological effective dose (BED) for early and late responding tissues for each protocol. The BED for one type of tissue is function of the dose per fraction and the number of fractions, and consequently the total dose, as well as the alpha/beta ratio of the evaluated tissue type (tumour or normal tissue). However, the basic BED calculation uses standard definitive protocols for models and does not take into account the length of treatment period and a correction factor should be included when the treatment time is reduced, like in case of accelerated protocols. Such a factor can be used with some specific knowledge about the tumour cell biology

(potential doubling time, cell loss factor) and an adjusted BED can then be calculated. However, an approximated BED (without adjustment) can be used when comparing protocols between them if the overall treatment time is similar. In presented report, the BED or the accelerated chemoradiotherapy protocol used is 66.15 Gy for early and 106.17 Gy for late responding tissues. Using a hypofractionated accelerated course with 10 fractions of 4.8 Gy (over 12 days) would give an approximated BED of 71.94 Gy for early and 124.6 Gy for late responding tissues (Hall, 2000). In that case, the potential for tumour control and acute side effects is possibly increased, yet the risk of delayed radiation-induced complications is certainly much higher and such a protocol might not be recommended if a long-term tumour control is possibly expected such as early stage tumours or more rostral oral SCC in dogs for instance.

The therapeutic benefit of adding chemotherapeutics as radiosensitizers to the radiotherapy treatments has been established for human squamous cell carcinomas at many tumour sites, including head and neck cancers, oesophageal, cervical and anal carcinomas (Apisarnthanarax et al., 2011). The biological mechanisms of interaction between chemotherapeutics and radiotherapy include interactions at molecular, cellular and tissue levels. Chemotherapeutics sensitise tumours to radiotherapy by inhibiting tumour cell repopulation, killing hypoxic cells, inhibiting the repair of sublethal radiation damage, sterilising micrometastatic disease in the radiation field and decreasing the tumour mass which leads to improved blood supply and reoxygenation (Argiris, 2002; Douple et al., 1985). When looking the recent data published in veterinary medicine the results of study of Petznek et al. (2014) supports the idea of using concomitant chemoradiotherapy instead of single treatment modalities. Although investigated on different tumour type cells and models, the authors proved *in vitro* (on feline injection site sarcomas derived primary tumour cell lines) and *in vivo* (on corresponding xenograft tumour mouse models) the efficacy of a concomitant chemo-/radiation therapy with doxorubicin resulting in a significant reduction in tumour growth compared to the respective monotherapies with either doxorubicin or radiation (Petznek et al., 2014).

All our patients received carboplatin, shown to have radiosensitization properties (Douple et al., 1985), along with radiation therapy, but the actual benefit in our cases remains unknown and any tumour control or survival improvement only hypothetical. As stated, chemotherapeutics can be used as radiosensitisers, however, the literature suggests that there is no effective chemotherapeutic agent, used alone, for the treatment of oral/pharyngeal or sino-nasal SCC. Several chemotherapeutic drugs were investigated with very limited success (Boria et al., 2004; Brooks et al., 1988; Buhles & Theilen, 1973; De Vos et al., 2005; Fox et al., 2000; Mauldin et al., 1988; Ogilvie et al, 1993). In veterinary medicine, beneficial effect of an accelerated chemoradiotherapy protocol (carboplatin used as a radiosensitizer) on the survival of cats with oral/tonsillar SCCs, was proved by Fidel and colleagues in comparison with accelerated radiotherapy protocol alone (Fidel et al., 2007; Fidel et al., 2011). Cisplatin combined with radiation, but not accelerated, for the treatment of canine nasal tumours did not improve survival times significantly (median survival time, 474 days) in comparison with other studies that used radiotherapy alone (Lana et al., 1997). However, chemotherapy can be a part of multimodal treatment approach that might help to improve the local tumour control and survival times. Additional chemotherapy is rarely warranted for rostral gingival SCC because of the low metastatic potential and it was not considered in the dog with rostral gingival SCC (Liptak & Withrow, 2007). However, it could be considered for SCC of the tongue, tonsil and caudal location of the mouth or sino-nasal SCC because of the higher metastatic rate. Only the owners of the dog with the tonsillar SCC decided for an additional 5 cycles of carboplatin chemotherapy (300 mg/m^2 every 3 weeks) in an attempt to prevent or delay further spread of the disease.

The treatment of advanced tumours affecting head and neck, especially those of tonsillar and sino-nasal origin, is difficult. Complex surrounding anatomical structures prevent treating the tumours surgically as clean margins are difficult to obtain. Radiation therapy is the only accepted treatment modality for the local control of canine sino-nasal tumours with median survival time ranging from 7 to 23 months (Adams et al., 2009; LaDue et al., 1999; Northrup et al., 2001; Theon et al., 1993). Although worse treatment outcome was expected for highly staged sino-nasal

tumours, the therapeutic benefit of accelerated chemoradiotherapy protocol in all, not only 4 sino-nasal cases, was confirmed. According to a study of Adams et al. (2009) the reported median survival for sino-nasal tumours with computed tomography evidence of cribriform plate involvement is 6.7 months. In the cases presented the treatment of 3 dogs with stage 4 sino-nasal SCC resulted in mean survival of 437 days indicating the beneficial effect of an accelerated chemoradiotherapy for this group of tumours affecting the head and neck.

We demonstrated that combining carboplatin as a radiosensitizer with twice daily radiotherapies performed could result in a highly effective loco-regional control rate as 3/3 patients with oral and tonsillar SCC achieved a complete tumour response. In all three patients, a complete disappearance of tumours was detected. All three dogs with sino-nasal SCC achieved a good treatment response. As CT performed 6 months and 1 year after finished chemoradiotherapy indicated a partial response in 1 dog and a complete response in 2 dogs with SNSCC. Only a dog with nasal planum SCC achieved a stable disease indicating this treatment protocol was not efficient enough to eliminate the disease. The overall survival of this dog was the shortest among all dogs treated. The explanation for this might be different and more aggressive biological behaviour of SCC of the nasal planum in comparison with other oral/tonsillar or sino-nasal SCC. The dog with nasal planum SCC was euthanised due to tumour progression while all other three dogs died or were euthanised due to tumour non-related causes. Organ preservation was achieved in all patients with oral/tonsillar SCC. An exception were three dogs with sino-nasal SCC that needed irradiated eye to be removed due to initial tumour effect and late radiotherapy side effects. Otherwise, none of the dogs had serious late side effects and all had/have reasonable quality of life 1-3 years after the completion of therapy and beyond. The mean overall survival for oral/tonsillar SCC was 32.4 months (972 days) and stage 4 sino-nasal SCC 12.9 months (389 days).

In this study, the incidence of acute toxicities were not worse as previously reported for standard definitive protocols except for a rather high incidence of oral mucositis that developed 2-3 weeks after finished treatment. However, all symptoms could be controlled by conservative treatment and diminished 5-6 weeks after treatment

completion. No detectable late complications have been observed with the protocol used 2.5 years later for dogs with oral/tonsillar SCC and 1 year after for dogs with sino-nasal SCC. Recurrence in such cases, despite achieving a complete response is common. Among canine patients with oral/tonsillar SCC only the dog with tonsillar SCC experienced recurrence in the regional lymph node 2 years after finished treatment with the primary site in a complete remission at the time of euthanasia. The local control of more than 1 year of duration with no evidence of tumour progression was also achieved in all three patients with sino-nasal SCC.

8 CONCLUSIONS

Firstly, we demonstrated significant differences in complete blood count indices (N/L, P/L, MPV/PLT and PLCRi) between healthy control dogs, dogs with stage 3,4 PD (moderate to advanced periodontitis) and dogs with neoplastic head and neck conditions. The N/L, P/L, MPV/PLT and PLCRi indices in dogs with neoplastic head and neck conditions were demonstrated to be significantly higher in comparison with the indices of healthy dogs and dogs with stage 3,4 PD (moderate to advanced periodontitis). Therefore we can suggest N/L, P/L, MPV/PLT and PLCRi indices as important distinguishing biomarkers between canine inflammatory and neoplastic head and neck conditions. Pre-treatment N/L, P/L, MPV/PLT and PLCRi were not confirmed as prognostic biomarkers in this group of dogs investigated.

Secondly, we have demonstrated that dogs with stage 3,4 PD (moderate to advanced periodontitis) exhibited significantly lower levels of peripheral blood and regional lymph node $CD4^+CD25^+$ and $CD4^+CD25^+FOXP3^+$ Tregs and other peripheral blood lymphocyte subpopulations ($CD4^+$, $CD8^+$, $CD5^+$ T cell-subsets and $CD21^+$ B cells) compared to dogs with stage 1 PD (gingivitis). The data obtained support the hypothesis of defects in the systemic level of Tregs in dogs with stage 3,4 PD (moderate to advanced periodontitis) which supports the idea of involvement of Tregs in the pathogenesis of periodontal disease with inflammatory and/or (auto)-immune background.

Thirdly, we have confirmed the hypothesis that the expression of Ki-67 and VEGFR-2 is higher in advanced canine neoplastic head and neck conditions. Canine sino-nasal SCC tumours demonstrated the highest expression of Ki-67 and VEGFR-2 in comparison with other neoplastic HN tumours, inflammatory and benign HN conditions, indicating on their more aggressive biologic behaviour. Malignant melanoma and sarcoma cases expressed less VEGFR-2 in comparison with sino-nasal SCC while oral SCC were almost always negative or expressed very low levels

of VEGFR-2. Increased expression of VEGFR-2, more specifically the IHC score >1 (more than 10% of malignant cells showing positive immunoreactivity) correlated with shorter overall survival.

Fourthly, the benefit of an accelerated chemoradiotherapy protocol in terms of amelioration of clinical signs, treatment response, progression free and overall survival in 6/7 canine patients with advanced SCCs of the head and neck was confirmed.

9 SUMMARY

The determination of the CBC indices (N/L, P/L, MPV/PLT and PLCRi) is after a review of the literature data the first study in veterinary medicine, carried out on healthy dogs and dogs with inflammatory and neoplastic head and neck conditions confirming significant differences in the CBC parameters and CBC indices between the groups of dogs investigated. It was shown that dogs with inflammatory and neoplastic head and neck processes have elevated N/L, P/L, MPV/PLT ratios and PLCRi in comparison with healthy dogs with differences found also between groups of dogs with different neoplastic head and neck tumours. We have demonstrated that differences between the CBC indices (N/L, P/L, MPV/PLT ratios and PLCRi) between healthy dogs and dogs with stage 3,4 PD (moderate to advanced periodontitis) exist but are much less pronounced than the differences between healthy dogs and dogs with neoplastic head and neck conditions. This suggests that PD in dogs is not causing considerable systemic effect as it was attributed to. We showed that CBC biomarkers (N/L, P/L, MPV/PLT ratios and PLCRi) can serve as inexpensive and readily available distinguishing biomarkers and important supportive biomarkers in the assessment of systemic inflammatory responses provoked by periodontal disease or benign and malignant head and neck tumours while the investigation of their prognostic value in dogs with neoplastic head and neck conditions was not confirmed and warrants further investigations.

Sufficient number and function of Tregs helps to maintain peripheral tolerance thus helping to prevent development of inflammatory and/or (auto)-immune mediated diseases, also PD. We have confirmed that dogs with stage 3,4 PD (moderate to advanced periodontitis) exhibited significant reduction of peripheral blood and regional lymph node Tregs ($CD4^+CD25^+$, $CD4^+CD25^+FOXP3^+$) and other lymphocyte subpopulations ($CD4^+$, $CD8^+$, $CD5^+$ T cell-subsets and $CD21^+$ B cells) in comparison with dogs with stage 1 PD (gingivitis) supporting the hypothesis that the mechanisms responsible for induction and maintenance of peripheral tolerance may

be disturbed with this type of inflammatory/infectious disease. We have confirmed that systemic alterations in the immune system, more specifically, in the systemic circulation of Tregs and other immunophenotype markers in dogs with stage 3,4 PD (moderate to advanced periodontitis) exist and may facilitate the multiplication, persistence and activity of periodontopathogens. Of course, further studies including assessment of biomarkers investigated ($CD4^+CD25^+$, $CD4^+CD25^+FOXP3^+$, $CD4^+$, $CD8^+$, $CD21^+$, $CD5^+$) in different clinical presentations of PD with following in a longer period of time are in progress to understand the exact relationship among Tregs and effector T cells regulating the balance between protective and destructive immune responses in the pathogenesis of periodontal diseases in dogs.

We have shown that VEGFR-2 status has the potential to be used as an important biomarker in diagnosis, prognosis and treatment of canine tumours arising from the head and neck. We confirmed the involvement of VEGFR-2 signalling pathway in canine malignant head and neck tumours by detecting the protein expression in 59% of cases. On the contrary, inflammatory and benign oral tissues were negative for VEGFR-2. Therefore, the potential use of VEGFR-2 as a supplemental diagnostic biomarker of malignancy affecting head and neck region in dogs can be suggested. It was found that oral SCC tumours were negative or expressed lower levels of VEGFR-2 in comparison with sino-nasal SCC tumours and this might offer a logical explanation for less aggressive behaviour of oral SCC. The concomitant high expression of Ki-67 and VEGFR-2 in sino-nasal SCC in comparison with oral SCC indicates on their more aggressive biologic behaviour in comparison with other head and neck tumours investigated. Malignant melanoma and sarcoma HN tumours expressed lower values of VEGFR-2 in comparison with sino-nasal SCC but higher in comparison with oral SCC. As dogs with head and neck tumours expressing higher percentage of VEGFR-2 had worse prognosis and shorter overall survival in comparison with those that did not express or expressed lower percentage of VEGFR-2 a negative prognostic significance of protein overexpression can be suggested.

The challenges in the treatment of canine sino-nasal head and neck tumours and advanced oral tumours with high percentage of tumour recurrences, have guided the research of treatment strategies including adjuvant therapies to improve the treatment outcome. One of the approaches to inhibit angiogenesis during tumorigenesis of head and neck tumours in dogs, expressing high percentage of VEGFR-2, could be the disruption of VEGF/VEGFR-2 pathways by VEGFR-2 inhibitors. Such drugs could suppress tumour growth by limiting their blood supply thus reducing uncontrolled neoplastic cell proliferation. Furthermore, many strategies used to radiosensitize tumours using biological targeted drugs have already been tested in human clinical trials and similar modulation may be useful in dogs with advanced malignant head and neck tumours, especially those of sino-nasal origin were surgical excision is not an option and where radiotherapy is the accepted treatment modality. Radiation therapy along with VEGFR-2 inhibitors may improve the treatment response of patients with head and neck tumours expressing VEGFR-2, especially those of sino-nasal origin.

The prospective nature of the fourth part of the study, presenting a single centre treatment experience demonstrated encouraging results in canine patient's outcomes following accelerated chemoradiotherapy. The overall treatment response of dogs receiving an accelerated chemoradiotherapy protocol can be assessed as good as the majority of dogs, an exception is a dog with nasal planum SCC, achieved a complete remission of more than 2-years in duration for advanced oral/tonsillar SCC and a good treatment response of more than 1-year for advanced sino-nasal SCC, suggesting that this chemo-radiotherapy protocol is a valuable alternative option in dogs with this type of neoplasia especially when taking into account tumour biology and owners needs like reduced costs and time requirements in contrast to the definitive protocols. Short and long-term side effects for the treatment of both oral/tonsillar and sino-nasal SCC were acceptable. As no standardized treatment protocol for these types of neoplasia exists at the moment, further progress in the management of head and neck SCC could be achieved by introduction of more advanced radiotherapy techniques with different fractionation schedules and/or

introduction of molecular targeted therapies in an attempt to reduce the incidence of loco-regional failure and to influence the improvement of disease free survival and overall survival rates. However, the role of the treatment approach presented needs further clarifications. The limitation of this study remains in a small number of patients suggesting an additional investigation of an accelerated radiotherapy protocols as well as the role of chemotherapeutics as radiosensitisers on a larger group of canine patients with HNSCC, especially those of tonsillar and sino-nasal origin.

10 POVZETEK

DIAGNOSTIČNI IN PROGNOSTIČNI MARKERJI PRI PSIH Z VNETNIMI IN NEOPLASTIČNIMI SPREMEMBAMI V PODROČJU GLAVE IN VRATU

Določanje indeksov krvne slike (N/L, P/L, MPV/PLT in PLCRi) je po pregledu literaturnih podatkov prva študija v veterinarski medicini, opravljena na zdravih psih in psih z vnetnimi in tumorskimi procesi v področju glave in vratu. Potrdili smo, da obstajajo razlike v krvnih parametrih in indeksih med preiskovanimi skupinami psov. Dokazali smo, da imajo psi z vnetnimi in neoplastičnimi procesi glave in vratu statistično značilno višje vrednosti N/L, P/L, MPV/PLT in PLCRi indeksov v primerjavi z zdravimi psi. Prav tako smo dokazali, da razlike med indeksi med zdravimi psi in psi s parodontalno boleznijo obstajajo, a so veliko manj izrazite kot razlike med zdravimi psi in psi s tumorskimi obolenji v področju glave in vratu. To nakazuje, da parodontalna bolezen pri psih verjetno ne povzroča tako izrazitega sistemskega vnetnega vpliva, kot se ji ga pripisuje. Dokazali smo, da indeksi krvne slike (N/L, P/L, MPV/PLT in PLCRi) lahko služijo kot hitro dostopni in poceni biomarkerji pri oceni sistemskih vnetnih odzivov izzvanih s parodontalno boleznijo, benignimi in malignimi tumorji v področju glave in vratu pri psih. Prognostična vrednost (N/L, P/L, MPV/PLT in PLCRi) pri psih z malignimi tumorskimi procesi glave in vratu ni bila dokazana.

Po literaturnih podatkih je zadostno število in primerna funkcija regulatornih T celic bistvenega pomena za ohranjanje periferne tolerance, preprečevanje razvoja avtoimunskih bolezni in omejevanje kroničnih vnetnih bolezni. Potrdili smo, da imajo psi z napredovalim periodontitisom statistično značilno zmanjšane vrednosti regulatornih T celic ($CD4^+CD25^+$, $CD4^+CD25^+FOXP3^+$) in drugih imunskih biomarkerjev ($CD4^+$, $CD8^+$, $CD5^+$ T celičnih subpopulacij in $CD21^+$ B celic) v periferni krvi in regulatornih T celic v področnih bezgavkah v primerjavi s psi s prvo stopnjo paradontalne bolezni (gingivitisom). Rezultati podpirajo hipotezo, da so mehanizmi za posredovanje uvajanja in vzdrževanja periferne tolerance moteni pri

tej vrsti vnetne bolezni. Potrdili smo, da spremembe v imunskem sistemu, natančneje v sistemski cirkulaciji regulatornih T celic in drugih imunskih biomarkerjih pri psih z napredovalim periodontitisom obstajajo in tako lahko prispevajo k razmnoževanju, obstoju in dejavnosti periodontopatogenih bakterij. Nadaljnje študije regulatornih T celic ($CD4^+CD25^+$, $CD4^+CD25^+FOXP3^+$) in drugih imunskih biomarkerjev ($CD4^+$, $CD8^+$, $CD5^+$ T celičnih subpopulacij in $CD21^+$ B celic) pri psih z različnimi kliničnimi stopnjami parodontalne bolezni in s sledenjem v daljšem časovnem obdobju, bodo omogočile natančnejše razumevanje vloge regulatornih in efektorskih T celic, ki urejajo ravnovesje med zaščitnimi in destruktivnimi imunskimi odzivi v patogenezi parodontalne bolezni pri psih.

Dokazali smo, da je VEGFR-2 lahko pomemben biomarker pri diagnozi, prognozi in zdravljenju tumorjev v področju glave in vratu pri psih. V 59% primerov malignih tumorjev glave in vratu pri psih smo dokazali izraženost VEGFR-2, medtem, ko je bila izraženost VEGFR-2 pri vnetnih ustnih lezijah in benignih ustnih tumorjih negativna ali zelo nizka. Na podlagi dobljenih rezultatov je VEGFR-2 lahko potencialni dodatni diagnostični biomarker malignosti pri psih s tumorji glave in vratu. Ugotovili smo, da je pri ustnih ploščato celičnih karcinomih (PCK) stopnja izraženosti VEGFR-2, v primerjavi s PCK nosnih in obnosnih votlin, nična ali zelo nizka in to lahko ponuja logično razlago za bolj agresivno biološko obnašanje slednjih. Sočasna visoka izraženost proliferacijskega biomarkerja Ki-67 in VEGFR-2 pri ploščatoceličnih tumorjih nosnih in obnosnih votlin v primerjavi z ustnimi PCK dodatno podpira dejstvo o njihovem agresivnem biološkem obnašanju v primerjavi z drugimi tumorji glave in vratu. Nižja izraženost VEGFR-2 je bila ugotovljena tudi pri malignem melanomu in sarkomu v primerjavi s PCK nosnih in obnosnih votlin. Dokazali smo prognostično vrednost VEGFR-2. Psi s PCK glave in vratu ter ostalimi malignimi tumorji z izraženim višjim deležem VEGFR-2 so imeli slabšo prognozo in krajše skupno preživetje v primerjavi s tistimi, pri katerih je bil odstotek izraženosti VEGFR-2 negativen oz. nižji.

Izzivi v zdravljenju PCK nosnih in obnosnih votlin kakor tudi sarkomov in ustnega malignega melanoma pri psih ostajajo, saj je pri le-teh odstotek pogostnosti

ponovitve bolezni oziroma pojava metastatske bolezni precej visok. Tumorji, pri katerih smo ugotovili višjo stopnjo izraženosti VEGFR-2 so potencialna tarča uporabe inhibitorjev, ki blokirajo VEGF/VEGFR-2 poti. Ta zdravila zavirajo rast tumorjev z omejevanjem dotoka krvi in s tem zmanjšujejo nenadzorovano proliferacijo tumorskih celic. Ta zdravila bi lahko delovala tudi kot radiosenzibilizirajoče substance predvsem pri psih z napredovalimi malignimi tumorji glave in vratu, kjer ostale možnosti terapije niso mogoče in kjer je radioterapija edini možni način zdravljenja. Radioterapevtsko zdravljenje v kombinaciji z inhibitorji VEGFR-2 poti bi v prihodnosti lahko izboljšalo odziv na zdravljenje pacientov s tumorji glave in vratu, pri katerih je ugotovljena izraženost VEGFR-2.

Večina psov, razen psa s ploščato-celičnim karcinomom (PCK) smrčka, je pri zdravljenju napredovalih oblik PCK v področju glave in vratu s popešenim protokolom obsevanja dosegla popoln odziv. Interval prost bolezni pri psih z napredovalimi PCK ustnimi tumorji je bil več kot 2 leti, medtem, ko je bil interval pri psih z napredovanimi PCK tumorji nosnih in obnosnih votlin več kot 1 leto. Kemoradioterapevtski protokol, prvič uporabljen in predstavljen za zdravljenje PCK ustne votline ter nosnih in obnosnih votlin pri psih, tako predstavlja alternativno možnost za zdravljenje psov s to vrsto tumorja. Če upoštevamo biologijo tumorja in potrebe lastnikov, kot so manjši stroški in čas potreben za izvedbo zdravljenja, v nasprotju z ostalimi standardnimi protokoli radioterapevtskega zdravljenja predstavlja novost. Akutni in kronični stranski učinki pri zdravljenju obeh vrst ploščato-celičnih karcinomov so bili sprejemljivi. Uvedba naprednejših radioterapevtskih tehnik s spreminjanjem frakcioniranja in/ali uvajanje bioloških zdravil ter uporaba kemoterapevtikov bi lahko dodatno prispevala k izboljšanju odziva na zdravljenje. Pomembno je doseči manjšo pojavnost področnih ponovitev bolezni in/ali razvoja metastaske bolezni kakor tudi vplivati na podaljšanje dobe preživetja pacientov s temi vrstami tumorjev.

11 REFERENCES

Aebershold, D.M., Burri, P., Beet, K.T., Laissue, J., Djonov, V., Greiner, R.H. and Semenza, G.L. (2001). Expression of hypoxia inducible factor-1 alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. *Cancer Research*, 61 (7), 2911-2916.

Abrams, V.K., Hwang, B., Lesnikova, M., Gass, M.J., Wayner, E., Castilla-Llorente C., Georges, G.E. and Nash, R.A. (2010). A novel monoclonal antibody specific for canine CD25 (P4A10): selection and evaluation of canine Tregs. *Veterinary Immunology and Immunopathology*, 135 (3-4), 257-265.

Adams, W.M., Withrow, S.J., Walshaw, R., Turrell, J.M., Evans, S.M., Walker, M.A. and Kurzman, I.D. (1987). Radiotherapy of malignant nasal tumours in 67 dogs. *Journal of the American Veterinary Medical Association*, 191 (3), 311-315.

Adams, W.M., Miller, P.E., Vail, D.M., Forrest, L.J. and MacEwen, E.G. (1998). An accelerated technique for irradiation of malignant canine nasal and paranasal sinus tumours. *Veterinary radiology ultrasound,* 39 (5), 475-481.

Adams, W.M., Bjorling, D.E., McAnulty, J.E. Green, E.M., Forrest, L.J. and Vail, D.M. (2005). Outcome of accelerated radiotherapy alone or accelerated radiotherapy followed by extenteration of the nasal cavity in dogs with intranasal neoplasia: 53 cases (1990-2002). *Journal of the American Veterinary Medical Association,* 227 (6), 936-941.

Adams, W.M., Kleiter, M.M., Thrall, D.E., Klauer, J.M., Forrest, L.J, La Due, T.A. and Havighurst, T.C. (2009). Prognostic significance of tumour histology and computed tomographic staging for radiation treatment response of canine nasal tumours. *Veterinary Radiology Ultrasound*, 50 (3), 330-335.

Agarwal, N., Kumar, V.S. and Gujjari, S.A. (2009). Effect of periodontal therapy on haemoglobin and erythrocyte levels in chronic generalized periodontitis patients: An interventional study. *Journal of Indian Society of Periodontology*, 13 (1), 6-11.

Albo, D., Granick, M.S., Jhala, N., Atkinson, B. and Solomon, M.P. (1994). The relationship of angiogenesis to biological activity in human squamous cell carcinomas of the head and neck. *Annals of Plastic Surgery*, 32 (6), 588-594.

Al-Dissi, A.N., Haines, D.M., Singh, B. and Kidney, B.A. (2007). Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor associated with tumour cell proliferation in canine cutaneous squamous cell carcinomas and trichoepitheliomas. *Veterinary Pathology*, 44 (6), 823-830.

Al-Dissi, A.N., Haines, D.M., Singh, B. and Kidney, B.A. (2009). Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor in canine cutaneous fibrosarcomas. *Journal of Comparative Pathology*, 141 (1), 229-236.

Al-Dissi, A.N., Haines, D.M., Singh, B. and Kidney, B.A. (2010). Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 in canine simple mammary gland adenocarcinomas. *Canadian Veterinary Journal*, 51 (1), 1109-1114.

Aljohani, H.A. (2010). Association between haemoglobin levels and severity of chronic periodontitis. *Scandinavian Journal of Medicine & Science in Sports*, 17(1), 53-64.

Allers, K., Loddenkemper, C., Hofmann, J., Unbehaun, A., Kunkel, D., Moos, V., Kaup, F.J., et al. (2010). Gut mucosal FOXP3+ regulatory CD4+ T cells and nonregulatory CD4+ T cells are differentially affected by simian immunodeficiency virus infection in rhesus macaques. *Journal of Virology*, 84 (7), 3259-3269.

Al-Rasheed, A. (2012). Elevation of white blood cells and platelet counts in patients having chronic periodontitis. *Saudi Dental Journal*, 24 (1), 17-21.

Amaya, H., Tanigawa, N., Lu, C., Matsumura, M., Shimomatsuya, T., Horiuchi, T. and Maraoka, R. (1997). Association of vascular endothelial growth factor expression with tumour angiogenesis, survival and thymidine phosphorylase/platelet-derived endothelial cell growth factor expression in human colorectal cancer. *Cancer Letters*, 119 (2), 227-235.

Ang, K.K., Peters, L.J., Weber, R.S., Maor, M.H., Morrison, W.H., Wendt, C.D. and Brown, B.W. (1990). Concomitant boost radiotherapy schedules in the treatment of carcinoma of the oropharynx and nasopharynx. *International Journal of Radiation Oncology Biology Physics*, 19 (6), 1339-1345.

Ang, K.K. (1988). Altered fractionation trials in head and neck cancer. *Seminars* in *Radiation* Oncology, 8 (4), 230-236.

Angelo, L.S. and Kurzrock, R. (2007). Vascular endothelial growth factor and its relationship to inflammatory mediators. *Clinical Cancer Research*, 13 (10), 2825-2830.

Anitua, E., Andia, I., Ardanza, B., Nurden, P. and Nurden, A.T. (2004). Autologous platelets as a source of proteins for healing and tissue regeneration. *Thrombosis* and *Haemostasis*, 91 (1), 4-15.

Anusaksathien, O., Singh, G., Matthews, N. and Dolby, A.E. (1992). Autoimmunity to collagen in adult periodontal disease: immunoglobulin classes in sera and tissue. *Journal of Periodontal Research,* 27 (1), 55-61.

Apisarnthanarax S, Dhruva N, Ardeshirpour F, Tepper J.E., Shores C.G., Rosenman J.G., et al. (2011). Concomitant radiotherapy and chemotherapy for high-risk nonmelanoma skin carcinomas of the head and neck. *International Journal of Surgical Oncology,* 464829, 8.

Argiris, A. (2002). Update on chemoradiotherapy for head and neck cancer. *Current Opinion in Oncology*, 14 (3), 323-329.

Armulik, A., Abramsson, A. and Betsholtz, C. (2005). Endothelial/pericyte interactions. *Circulation Research*, 97 (6), 512-523.

Artese, L., Simon, M.J., Piattelli, A., Ferrari, D.S., Cardoso, L.A., Faveri, M., et al. (2011). Immunohistochemical analysis of inflammatory infiltrate in aggressive and chronic periodontitis: a comparative study. *Clinical Oral Investigations,* 15 (2), 233-240.

Arzi, B. & Verstraete, F.J.M. (2012). Clinical staging and biopsy of maxillofacial tumours. In: Verstraete FJM, Lommer MJ, Bezuidenhout AJ, editors. Oral and Maxillofacial Surgery in Dogs and Cats. Edinburgh: Saunders/Elsevier; pp 373-380.

Asano, M., Toda, M., Sakaguchi, N. and Sakaguchi, S. (1996). Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *Journal of Experimental Medicine*, 1, 184 (2), 387-396.

Asher, V., Lee, J., Innamaa, A. and Bali, A. (2011). Preoperative platelet to lymphocyte ratio as an independent prognostic marker in ovarian cancer. *Clinical and Translational Oncology*, 13 (7), 499-503.

AVDC (American Veterinary Dental College). (2012). Nomenclature adopted by the AVDC Board. Periodontal disease classification. Available from http://www.avdc.org/nomenclature. Accessed 20/10/2015.

Azab, B., Bhatt, V.R., Phookan, J., Murukutla, S., Kohn, N., Terjanin, T. and Widmann, W.D. (2012). Usefulness of the neutrophil to lymphocyte ratio in predicting short and long-term mortality in breast cancer patients. *Annals of Surgical Oncology*, 19(1): 217-224.

Baecher-Allan, C., Brown, J.A., Freeman, G.J. and Hafler, D.A. (2001). CD4+CD25 high regulatory cells in human peripheral blood. *Journal of Immunology*, 167 (3), 1245-1253.

Baecher-Allan, C., Wolf, E. and Hafter, D.A. (2006) MHC class II expression identifies functionally distinct human regulatory T cells. *Journal of Immunology*; 176(8): 4622-4631.

Balkwill, F. and Mantovani, A. (2001). Inflammation and cancer: back to Wirchow? *Lancet*, 357 (9255), 539-545.

Balta, S., Demirkol, S., Unlu, M., Arslan, Z. and Celik, T. (2013). Neutrophil to lymphocyte ratio may be predict of mortality in all conditions. *British Journal of Cancer*, 09 (12), 3125-3126.

Bambace, N.M. and Holmes, C.E. (2011). The platelet contribution to cancer progression. *Journal of Thrombosis and Haemostasis,* 9 (2), 237-249.

Bateman, K.E., Catton, P.A., Pennock, P.W. and Kruth, S.A. (1994). 0-7-21 radiation therapy for the treatment of canine oral melanoma. *Journal* of *Veterinary Internal Medicine*, 8 (4), 267-272.

Bath, P.M. and Butterworth, R.J. (1996). Platelet size: measurement, physiology and vascular disease. *Blood Coagulation and Fibrinolysis*, 7 (2), 157-161.

Beissert, S., Schwarz, A. and Schwarz, T. (2006). Regulatory T cells. *Journal of Investigative Dermatology*, 126 (1), 15-24.

Beikler, T. and Flemmig, T.F. (2011). Oral biofilm-associated diseases: trends and implications for quality of life, systemic health and expenditures. *Periodontology 2000*, 55 (1), 87-103.

Belkaid, Y. and Rouse, B.T. (2005). Natural regulatory T cells in infectious disease. *Nature Immunology*, 6 (4), 353-360.

Belkaid, Y. and Tarbell, K. (2009). Regulatory T cells in the control of host microorganism interactions. *Annual Review of Immunology*, 27, 551-589.

Benham, A.H., Lyne, L., Scase, T.J. and Blacklaws, B.A. (2009). Monoclonal antibodies raised to the human FOXP3 protein can be used effectively for detecting Foxp3(+) T cells in other mammalian species. *Veterinary Immunology and Immunopathology*, 127 (3-4), 376-381.

Bennett, C.L., Christie J., Ramsdell F., Brunkow M.E., Ferguson P.J., Whitesell L., et al. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature Genetics*, 27 (1), 20-21.

Bentzen S.M. (2003). Repopulation in radiation oncology: perspectives of clinical research. *International Journal of Radiation Biology*, 79 (7), 581-585.

Bergers, G. and Benjamin, L.E. (2003). Tumorigenesis and the angiogenic switch. *Nature Reviews Cancer,* 3 (6), 401-410.

Bergers, G. and Hanahan, D. (2008) Modes of resistance to anti-angiogenic therapy. *Nature Reviews Cancer*, 8 (8), 592-603.

Bergin, I.L., Smedley, R.C., Esplin, D.G., Spangler, W.L. and Kiupel, M. (2011). Prognostic Evaluation of Ki67 threshold value in canine oral melanoma. *Veterinary Clinical Pathology*, 48 (1), 41-53.

Bergkvist, G.T., Argyle, D.J., Morrison, L., MacIntyre, N., Hayes, A. and Yool, D.A. (2011). Expression of epidermal growth factor receptor (EGFR) and Ki67 in feline oral squamous cell carcinomas (FOSCC). *Veterinary and Comparative Oncology*, 9 (2), 106-117.

Berglundh, T., Donati, M. and Zitzmann, N. (2007). B cells in periodontitis: Friends or enemies? *Periodontology 2000*, 45: 51-66.

Bergman, P.J. and Wolchok, J.D. (2008). Of mice and men (and dogs): development of a xenogeneic DNA vaccine for canine oral malignant melanoma. *Journal of Cancer Therapy*, 6, 817-826.

Bettelli, E., Dastrange, M. and Oukka, M. (2005). Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proceedings of the National Academy of Sciences of the United States of America,* 102 (14), 5138-5143.

Beyer, M. and Schutze, J.L. (2006). Regulatory T cells in cancer. *Blood*, 108, 804-811.

Bhansali, R.S., Yeltiwar, R.K. and Bhat, K.G. (2013). Assessment of peripheral neutrophil functions in patients with localized aggressive periodontitis in the Indian population. *Journal of Indian Society of Periodontology*, 17 (6), 731-736.

Bhatti, I., Peacock, O., Lloyd, G., Larvin, M. and Hall, R.I. (2010). Preoperative hematologic markers as independent predictors of prognosis in resected pancreatic ductal adenocarcinoma: neutrophil-lymphocyte versus platelet-lymphocyte ratio. *American Journal of Surgery***,** 200 (2), 197-203.

Biller, B.J., Elmslie, R.E., Burnett, R.C., Avery, A.C. and Dow, S.W. (2007). Use of FOXP3 expression to identify regulatory T cells in healthy dogs and dogs with cancer. *Veterinary Immunology and Immunopathology*, 116 (1-2), 69-78.

Biller, B.J., Guth, A., Burton, J.H. and Dow, S.W. (2010). Decreased ratio of CD8+ T cells to regulatory T cells associated with decreased survival in dogs with osteosarcoma. *Journal of Veterinary Medicine*, 24 (5), 1118-1123.

Bishop, J.M. (1987). The molecular genetics of cancer. *Science*, 235 (4786): 305-311.

Bittner, M.I. and Grosu, A.L. (2013). Hypoxia in head and neck tumours. Characteristics and development during therapy. Mini Review article. Frontiers in Oncology, 3: 223.

Blackwood, L. and Dobson J.M. (1996). Radiotherapy of oral malignant melanomas in dogs. *Journal of the American Veterinary Medical Association*, 209 (1), 98-102.

Bluestone, J.A. and Abbas, A.K. (2003). Natural versus adaptive regulatory T cells. *Nature Reviews Immunology*, 3 (3), 253-257.

Boer, M.C., Joosten, S.A. and Otenhoff, T.H. (2015). Regulatory T cells at the interface between human host and pathogens in infectious diseases and vaccination. *Frontiers in Immunology*, 6: 217.

Bolacchi, F., Sinistro, A., Ciaprini, C., Demin, F., Capozzi, M., Carducci, F.C., et al. (2006). Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4+ T cell response in HCV-infected

patients with normal versus abnormal alanine aminotransferase levels. *Clinical and Experimental Immunology*, 144 (2), 188-196.

Bolzer, K., Kaser, T., Saalmueller, A. and Hammer, S.E. (2009). Molecular characterisation of porcine forkhead-box p3 (Foxp3). *Veterinary Immunology and Immunopathology*, 132 (2-4), 275-281.

Boria, P.A., Murry, D.Y., Bennet, P.F., Glickman, N.W., Snyder, P.W., Merkel, B.L., et al. (2004). Evaluation of cisplatin combined with piroxicam for the treatment of oral malignant melanoma and squamous cell carcinoma in dogs. *Journal of the American Veterinary Medical Association,* 224 (3) 388-394.

Bosari, S., Lee, A.K., DeLellis, R.A., Wiley, B.D., Heatley, G.J. and Silverman, M.L. (1992). Microvessel quantitation and prognosis in invasive breast carcinoma. *Human Pathology*, 23 (7), 755-761.

Bourhis, J., Lapeyre, M., Tortochaux, J., Rives, M., Aghili, M., Bourdin, S., et al. (2006). Phase III randomized trial of very accelerated radiation therapy compared with conventional radiation therapy in squamous cell head and neck cancer: A GORTEC trial. *Journal of Clinical Oncology,* 24 (18), 2873-2878.

Bourhis, J., Overgaard, J., Audry, H., Ang, K.K., Saunders, M., Bernier, J., et al. (2006). Meta-Analysis of radiotherapy in carcinomas of head and neck (MARCH) collaborative group: Hyperfractionated or accelerated radiotherapy in head and neck cancer: A meta-analysis. *Lancet,* 368 (9538), 843-854.

Brana, I. and Siu, L.L. (2012). Locally advanced head and neck squamous cell cancer: treatment choice based on risk factors and optimazing drug prescription. *Annals of Oncology*, 23 (10), 178-185.

Brandau, S., Dumitru, C.A. and Lang, S. (2012). Protumor and antitumor functions of neutrophil granulocytes. Seminars in Immunopathology, 35, 163-176.

Brock, C.S. & Lee, S.M. (2002). Antiangiogenic strategies and vascular targeting in the treatment of lung cancer. European Journal of Respiratory Diseases, 19 (3), 557-570.

Brooks, M.B., Matus, R.E., Leifer, C.E., Alfieri, A.A. and Patnaik, A.K. (1988) Chemotherapy versus chemotherapy plus radiotherapy in the treatment of tonsillar squamous cell carcinoma in the dog. *Journal of Veterinary Internal Medicine*, 2 (4), 206-211.

Brown, A.S., Hong, Y., de Belder, A., Beacon, H., Beeso, J., Sherwood, R., et al. (1997). Megakaryocyte ploidy and platelet changes in human diabetes and atherosclerosis. *Arteriosclerosis*, *Thrombosis* and *Vascular Biology*, 17 (4), 802-807.

Brown, L.F., Berse, B., Jackman, R.W., Tognazzi, K., Manseau, E.J., Dvorak, H.F. and Senger, D.R. (1993). Increased expression of vascular permeability factor

(vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. *American Journal of Pathology*, 143 (5), 1255-1262.

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Papeper, B., Clark, L.B., Yasayko, S.A., et al. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics*, 27 (1), 68-73.

Buckner, J.H. (2010). Mechanisms of impaired regulation by CD4+CD25+FOXP3+ regulatory T cells in human autoimmune diseases. *Nature Reviews Immunology*, 10 (12), 849-859.

Budach, W., Hehr, T., Budach, V., Belka, C. and Dietz, K. (2006). A meta-analysis of hyperfractionated and accelerated radiotherapy and combined chemotherapy and radiotherapy regimens in unresected locally advanced squamous cell carcinoma of the head and neck. *BMC Cancer,* 6, 28.

Buhles, W.R. and Theilen, G.H. (1973). Preliminary evaluation of bleomycin in feline and canine squamous cell carcinoma. *American Journal of Veterinary Research*, 34 (2), 289-291.

Buhlin, K., Mäntylä, P., Paju, S., Peltola, J.S., Nieminen, M.S., Sinisalo, J. and Pussinen, P.J. (2011). Periodontitis is associated with angiographically verified coronary artery disease. *Journal of Clinical Periodontology*, 38 (11), 1007-1014.

Cannon, C.P., McCabe, C.H., Wilcox, T.G., Bentley, J.H. and Braunwald, E. (2001). Association of white blood cell count with increased mortality in acute mayocardial infarction and unstable angina pectoris. *American Journal of Cardiology*, 87 (5), 636-639.

Cardoso, C.R., Garlet, G.P., Moreira, A.P., Junior, W.M., Rossi, M.A. and Silva, JS. (2008). Characterization of CD4+CD25+ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis. *Journal of Leukocyte Biology*, 84 (1), 311-318.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*, 380 (6573), 435-439.

Carmeliet, P. (2003). Angiogenesis in health and disease. *Journal of Natural Medicines*, 9 (6), 653-660.

Catalano, P. (2002). White blood cell count with differential. In: George-Gay B, Chernecky C (Eds.): Clinical Medical-Surgical Nursing, Philadelphia, PA, Saunders, pp 282-290.

Cavassani, K.A., Campanelli, A.P., Moreira, A.P., Vancim, J.O., Vitali, L.H., Mamede, R.C., et al. (2006). Systemic and local characterization of regulatory T

cells in a chronic fungal infection in humans. *Journal of Immunology*, 177 (9), 5811-5818.

Cedres, S., Torrejon, D., Martinez, A., Martinez, P., Nevarro, A., Zamora, E., et al. (2012). Neutrophil to lymphocyte ratio (NLR) as an indicator of poor prognosis in stage IV non-small cell lung cancer. *Clinical and Translational Oncology*, 14 (11), 864-869.

Celenligil, H., Kansu, E., Ruacan, S., Eratalay, K. and Caglayan, G. (1993). In-situ characterization of gingival mononuclear cells in rapidly progressive periodontitis. *Journal of Periodontology*, 64 (2), 120-127.

Chai, L., Ying, H.F., Wu, T.T., Zhou, S.H., Bao, Y.Y., Yao, H.T. and You, Q.H. (2014). Clinical features and hypoxic marker expression of primary sinonasal and laryngeal small-cell neuroendocrine carcinoma: a small case series. *World Journal of Surgical Oncology*, 12: 199.

Chatila, T.A., Blaeser, F., Ho, N., Lederman, H.M., Voulgaropoulos, C., Helms, C. and Bowcock, AM. (2000). JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *The Journal of Clinical Investigation*, 06 (12), R75-81.

Chavele, K.M. and Ehrenstein, M.R. (2011). Regulatory T cells in systemic lupus erythematosus and rheumatoid arthritis. *Federation of European Biochemical Societies Letter*, 1-585 (23), 3603-3610.

Cho, S.Y., Yang, J.J., You, E., Kim, B.H., Shim, J., Lee, H.J, et al. (2013). Mean platelet volume/platelet count ratio in hepatocellular carcinoma. *Platelets*, 24 (5), 375-377.

Cifcibasi, E., Ciblak, M., Kiran, B., Badur, S., Firatli, E., Issever, H. and Cintan, S. (2015). The Role of Activated Cytotoxic T Cells in Etiopathogenesis of Periodontal Disease: Does It Harm or Does It Heal? *Scientific Reports*, 5, 9262.

Clark, R., and Kupper, T. (2005). Old meets new: the interaction between innate and adaptive immunity. *Journal of Investigative Dermatology*, 125, 629-637.

Cohen, J. (1988). Statistical Power Analysis for the Behavioral Sciences. 2nd ed. Mahwah, New Jersey: Lawrence Erlbaum; Differences between correlation coefficients; pp. 109-139.

Coffin, J.D. and Poole, T.J. (1988). Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel promordia in quail embryos. *Development*, 102 (4), 735-748.

Collet, G., Lamerant-Fayel, N., Tertil, M., El Hafny-Rahbi, B.E., Stepniewski, J., Guichard, A., et al. (2014). Hypoxia-regulated over expression of soluble VEGFR2 controls angiogenesis and inhibits tumour growth. *Molecular Cancer Therapeutics*, 13 (1), 165-178.

Colombo, N.H., Shirakashi, D.J., Chiba, F.Y., Coutinho, M.S., Ervolino, E., Garbin, C.A., et al. (2012). Periodontal disease decreases insulin sensitivity and insulin signalling. *Journal of Periodontology,* 83 (7), 864-870.

Cools, N., Ponsaerts, P., Van Tendeloo, V.F. and Berneman, Z.N. (2007). Regulatory T cells and human disease. *Clinical and Developmental Immunology*, 2007: 891-895.

Cooper, M.D. and Alder, M.N. (2006). The evolution of adaptive immune systems. *Cell*; 124 (4), 815-822.

Corvo, R. (2007). Evidence-based radiation oncology in head and neck squamous cell carcinoma. *Radiotherapy & Oncology*; 85 (1), 156-170.

Costa, C., Incio, J. and Soares, R. (2007). Angiogenesis and chronic inflammation: cause or consequence? *Angiogenesis*, 10 (3), 149-166.

Costantini, V., Zacharski, L.R., Moritz, T.E. and Edwards, R.L. (1990). The platelet count in carcinoma of the lung and colon. *Thrombosis and Haemostasis*, 64 (4), 501-505.

Costantino, C.M., Baecher-Allan, C.M. and Hafler, D.A. (2008). Human regulatory T cells and autoimmunity. *European Journal of Immunology,* 38 (4), 921-924.

Coussens, L.M. and Werb, Z. (2002). Inflammation and cancer. *Nature*, 420 (6917), 860-867.

Couture, C., Raybaud-Diogene, H., Tetu, B., Bairati, I., Murry, D., Allard, J. and Fortin, A. (2002). p53 and Ki-67 as markers of radioresistance in head and neck carcinoma. *Cancer,* 94 (3), 713-722.

Cray, C. (2012). Acute phase proteins in animals. *Molecular Biology and Translational Science,* 105, 113-150.

D'Aiuto, F., Parker, M., Andreou, G., Suvan, J., Brett, P.M., Raedy, D. and Tonetti, M.S. (2004). Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers. *Journal of Dental Research*, 83 (2), 156-60.

D'Aiuto, F., Parkar, M., Nibali, L., Suvan, J., Lessem, J. and Tonetti, M.S. (2006). Periodontal infections cause changes in traditional and novel cardiovascular risk factors: Results from a randomized controlled clinical trial. *American Heart Journal*, 151 (5), 977-984.

Dameron, K.M., Volpert, O.V., Tainsky, M.A. and Bouck, N. (1994). Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin 1. *Science*, 265 (5178), 1582-1584.

Damert, A., Machein, M., Breier, G., Fujita, M.Q., Hanahan, D., Risau, W. and Plate, K.H. (1997). Up-regulation of vascular endothelial growth factor expression in a rat

glioma is conferred by two distinct hypoxia-driven mechanisms. *Cancer Research*, 57 (17), 3860-3864.

Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., et al. (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *Journal of Experimental Medicine*, 204 (6), 1257–1265.

DeBowes, L.J., Moiser, D., Logan, E., Harvey, C.E., Lowry, S., Richardson, D.C. (1996). Association of periodontal disease and histologic lesions in multiple organs from 45 dogs. *Journal of Veterinary Dentistry*, 13 (2), 57-60.

DeBowes, L.J. (1998). The effects of dental disease on systemic disease. *Veterinary Clinics of North America: Small Animal Practice,* 28 (5), 1057-1062.

De-Gennaro, L.A., Lopes, J.D. and Mariano, M. (2006). Autoantibodies directed to extracellular matrix components in patients with different clinical forms of periodontitis. *Journal of Periodontology*, 77 (12), 2025-2030.

De Larco, J.E., Wuertz, B.R. and Furcht, L.T. (2004). The potential role of neutrophils in promoting the metastatic phenotype of tumours releasing interleukin-8. *Clinical Cancer Research,* 10 (15), 4895-4900.

De Luca, G., Santagostino, M., Secco, G.G., Cassetti, E., Giuliani, L., Coppo, L., et al. (2010). Platelet large cell ratio in the extent of coronary artery disease: results from a large prospective study. *Journal of thrombosis and thrombolysis*, 30 (4), 426-433.

DeNardo, D.G., Andreau, P. and Coussens, L.M. (2010). Interactions between lymphocytes and myeloid cells regulate pro-versus anti-tumour immunity. *Cancer and Metastasis Reviews*, 29 (2), 309-316.

Denhart, B.C., Guidi, A.J., Tognazzi, K., Dvorak, H.F. and Brown, L.F. (1997). Vascular permeability factor/vascular endothelial growth factor and receptors in oral and larynegal squamous cell carcinoma and dysplasia. *Laboratory Investigation*, 77 (6), 659-664.

Denijn, M. and Ruiter, D.J. (1993). The possible role of angiogenesis in the metastatic potential of human melanoma. Clinicopathological aspects. *Melanoma Research,* 3 (1), 5-14.

Detmar, M., Brown, L.F., Claffey, K.P., Yeo, K.T., Kocher, O., Jackman, R.W., et al. (1994). Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *Journal of Experimental Medicine*, 180 (3), 1141-1146.

De Vos, J.P., Burm, A.G., Focker, A.P., Boschloo, H., Karsijns, M. and van der Waal, I. (2005). Piroxicam and carboplatin as a combinant treatment of canine oral non-tonsillar squamous cell carcinoma: a pilot study and a literature review of canine

model of human head and neck squamous cell carcinoma. *Veterinary and Comparative Oncology*, 3 (1), 16-24.

Dicato, M., Plawny, L. and Diederich, M. (2010). Anemia in cancer. *Annals of Oncology*, 21 Suppl 7.

Dobrossy, L. (2005). Epidemiology of head and neck cancer: magnitude of the problem. *Cancer and Metastasis Reviews*, 24 (1), 9-17.

Donnem, T., Al-Shibli, K., Andersen, S., Al-Saad, S., Busund, L.T. and Bremnes, R.M. (2010). Combination of low vascular endothelial growth factor A (VEGF-A/VEGF receptor 2 expression in high lymphocyte infiltration is a strong and independent prognostic factor in patients with non-small cell lung cancer. *Cancer*, 116 (18): 4318-4325.

Donskov, F. (2013). Immunomonitoring and prognostic relevance of neutrophils in clinical trials. *Seminars in Cancer Biology*, 23 (3), 200-207.

Douple, E.B., Richmond, R.C., O'Hara, J.A. and Coughlin, C.T. (1985). Carboplatin as a potentiator of radiation therapy. *Cancer Treatment Reviews*, 12, 111-124.

Du, J.R., Jiang, Y., Zhang, Y.M. and Fu, H. (2003). Vascular endothelial growth factor and microvascular density in oesophageal and gastric carcinomas. *World Journal of Gastroenterology*, 9 (7), 1604-1606.

Duarte, P.M., Santos, V.R., Dos Santos, F.A., de Lima Pereira, S.A., Rodrigues, D.B. and Napimoga, MH. (2011). Role of smoking and type 2 diabetes in the immunobalance of advanced chronic periodontitis. *Journal of Periodontology*, 82 (3), 429-438.

Dubernard, V., Arbeille, B.B., Lemesle, M.B. and Legrand, C. (1997). Evidence of alpha granular pool of cytoskeletal protein alpha-actinin in human platelets that redistributes the adhesive glycoprotein thrombospodonin 1 during the exyocytotic process. *Arteriosclerosis Thrombosis and Vascular Biology*, 17 (19), 2293-2305.

Dutzan, N., Gamonal, J., Silva, A., Sanz, M. and Vernal, R. (2009). Over-expression of forkhead box P3 and its association with receptor activator of nuclear factor-kappa B ligand, interleukin (IL)-17, IL-10 and transforming growth factor-beta during the progression of chronic periodontitis. *Journal of Clinical Periodontology*, 36 (5), 396-403.

Dvorak, H.F., Brown, L.F., Detmar, M. and Dvorak, A.M. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability and angiogenesis. *The American Journal of Pathology***,** 1995; 146(5): 1029-1039.

Ebersole, J.L. and Taubman, M.A. (1994). The protective nature of host responses in periodontal diseases. *Periodontology 2000*, 5, 112-141.

Ebersole, J.L., Machen, R.L., Steffen, M.J. and William, D.E. (1997). Systemic acute phase reactants, C-reactive protein and heptoglobin in adult periodontitis. *Clinical Experimental Immunology*, 107 (2), 347-352.

Egan K., Crowley D., Smyth P., O'Toole S., Spillane C., Martin C., et al. (2011). Platelet adhesion and degranulation induce pro- survival and pro-angiogenic signal lining in ovarian cancer cells. *Plos One,* 6 (10), 26125.

Eisma, R.J., Spiro, J.D. and Kreuzer, D.L. (1997). Vascular endothelial growth factor expression in head and neck squamous cell carcinoma. *American Journal of Surgery*, 174 (5), 513-517.

Elinav, E., Nowarski, R., Thaiss, C.A., Hu, B., Jin, C. and Flavell, R.A. (2013). Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nature Reviews Cancer,* 13(11), 759-771.

Ellis, J.A. and Demartini, J.C. (1985). Ovine concanavalin A-induced suppressor cells: generation, assay, age-related effects and re-evaluation of mechanism of suppression. *Immunology*, 54 (2), 353-362.

Engan, T. and Hannisdal, E. (1990). Blood analyses as prognostic factors in primary lung cancer. *Acta Oncologica*, 29 (2), 151-154.

Engel, D., Monzingo, S., Rabinovitch, P., Clagett, J. and Stone, R. (1984). Mitogen-induced hyperproliferaton response of peripheral blood mononuclear cells from patients with severe generalized periodontitis: lack of correlation with proportions of T cells and T-cell subsets. *Clinical Immunology and Immunopathology*, 30 (3): 374-386.

Erciyas, K., Orbak, R., Kavrut, F., Demir, T. and Kaya, H. (2006). The changes in T lymphocyte subsets following periodontal treatment in patients with chronic periodontitis. *Journal of Periodontal Research,* 41 (3), 165-170.

Evans, S.M., Goldschmidt, M., McKee, L.J. and Harvey, C.E. (1989). Prognostic factors and survival after radiotherapy for intranasal neoplasms in dogs: 70 cases (1974-1985). *Journal of American Veterinary Medical Association,* 194, 1460-1463.

Fan, F., Wey, J.S., McCarty, M.F., Belcheva, A., Liu, W., Bauer, T.W., et al. (2005). Expression and function of vascular endothelial growth factor receptor 1 on human colorectal cancer cells. *Oncogene,* 24, 2647-2653.

Farber, E. (1984). The multistep nature of cancer development. *Cancer Research,* 44 (10), 4217-4223.

Fava, R.A., Olsen, N.J., Spencer-Green, G., Yeo, K.T., Yeo, T.K., Berse, B., et al. (1994). Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. *Journal of Experimental Medicine*, 180 (1), 341-346.

Feng, J., Wang, Z., Guo, X., Chen, Y., Cheng, Y. and Tang, Y. (2012). Prognostic significance of absolute lymphocyte count at diagnosis of diffuse large B-cell lymphoma: a meta-analysis. *International Journal of Haematology*, 95 (2), 143-148.

Ferrara, N. (1999). Vascular endothelial growth factor: molecular and biological aspects. *Current Topics in Microbiology and Immunology*, 237, 1-30.

Ferrara, N., Gerber, H.P. and LeCouter, J. (2003). The biology of VEGF and its receptors. *Nature Medicine*, 9 (6), 669-676.

Ferrara, N. (2004). Vascular endothelial growth factor: basic science and clinical progress. *Endocrine Reviews*, 25 (4), 581-611.

Ferrara, N. (2009). VEGF-A: a critical regulator of blood vessel growth. *European Cytokine Network*, 20 (4), 158-163.

Ferrara, N. and Davis-Smyth, T. (1997). The biology of vascular endothelial growth factor. *Endocrine Reviews***,** 18 (1), 4-25.

Feuerer, M., Hill, J.A., Mathis, D. and Benoist, C. (2009). Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nature Immunology*, 10 (7), 689-695.

Fidel, J.L., Sellon, R.K., Houston, R.K. and Wheeler, B.A. (2007). A nine-day accelerated radiation protocol for feline squamous cell carcinoma. *Veterinary Radiology & Ultrasound*, 48(5), 482- 485.

Fidel J., Lyons J., Tripp C., Houston R., Wheeler B. and Ruiz A. (2011). Treatment of oral squamous cell carcinoma with accelerated radiation therapy and concomitant carboplatin in cats. *Journal of Veterinary Internal Medicine*, 25 (3), 504-510.

Fischer, C.A., Jung, M., Zlobec, I., Green, E., Storck, C., Tornillo, L., et al. (2011). Co-expression of p21 and Ki-67 in head and neck squamous cell carcinoma relative to a significantly poor prognosis. *Head & Neck*, 33 (2), 267-273.

Flavell, R.A., Sanjabi, S., Wrzesinski, S.H. and Licona-Limón, P. (2010). The polarization of immune cells in the tumour environment by TGFβ. *Nature Reviews Immunology*, 10 (8), 554–567.

Flickinger, I., Tutgen, B.C., Gerner, W., Calice, I., Tichy, A., Saalamuller, A. and Kleiter, M. (2013). Radiation up-regulates the expression of VEGF in canine oral melanoma cell line. *Journal of Veterinary Science*, 14 (2), 207-214.

Folkman, J. (1990). What is the evidence that tumours are angiogenesis dependent? *Journal of the National Cancer Institute,* 82 (1), 4-6.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Natural Medicine,* 1 (1), 27-31.

Folkman, J. (2007). Angiogenesis: an organizing principle for drug discovery? *Nature Reviews Drug Discovery*, 6 (4), 273-286.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D. and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Molecular and Cellular Biology*, 16 (9), 4604-4613.

Foussat, A., Cottrez, F., Brun, V., Fournier, N., Breittmayer, J.P. and Groux, H. (2003). A comparative study between T regulatory type 1 and CD4+CD25+ T cells in the control of inflammation. *Journal of Immunology*, 171 (10), 5018-5026.

Fox, L.E., Rosenthal, R.C., King, R.R., Levine, P.B., Vail, D.M., Helfand, S.C., et al. (2000). Use of cis-bis-neodecanoato-trans-R,R-1, 2-diaminocyclohexane platinum (II), a liposomal cisplatin analogue, in cats with oral squamous cell carcinoma. *American Journal of Veterinary Research,* 61 (7), 791-795.

Freeman, K.P., Hahn, K.A., Harris, F.D. and King, G.K. (2003). Treatment of dogs with oral melanoma by hypofractionated radiation therapy and platinum-based chemotherapy (1987-1997). *Journal of Veterinary Internal Medicine*, 17 (1), 96-101.

Freudlsperger, C., Freier, K., Hoffmann, J. and Engel, M. (2012). Ki-67 expression predicts radiosensitivity in oral squamous cell carcinoma. *International Journal of Oral and Maxillofacial Surgery*, 41 (8), 965-969.

Frey, O. and Bräuer, R. (2006). Regulatory T cells: magic bullets for immunotherapy? *Archivum Immunologiae et Therapiae Experimentalis (Warsz),* 54 (1), 33-43.

Fridlender, Z.G. and Albeida, S.M. (2012). Tumour associated neutrophils: friend or foe? *Carcinogenesis,* 33 (5), 949-955.

Fu, K.K., Clery, M., Ang, K.K., Byhardt, R.W., Maor, M.H. and Beitler, J.J. (1995). Randomized phase I/II trial of two variants of accelerated fractionated radiotherapy regimens for advanced head and neck cancer: Results of RTOG 88-09. *International Journal of Radiation Oncology Biology Physics,* 32 (3), 589-597.

Fu, K.K., Pajak, T.F., Trotti, A., Jones, C.U., Spencer, S.A., Phillips, T.L., et al. (2000). A Radiation Therapy Oncology Group (RTOG) phase III randomized study to compare hyperfractionation and two variants of accelerated fractionation to standard fractionation radiotherapy for head and neck squamous cell carcinomas: First report of RTOG 9003. *International Journal of Radiation Oncology Biology Physics*, 48 (1), 7-16.

Fu, D.R., Kato, D., Watabe, A., Endo, Y. and Kadosawa, T. (2014). Prognostic utility of apoptosis index, Ki-67 and survin expression in dogs with nasal carcinoma treated with orthovoltage radiation therapy. *Journal of Veterinary Medical Science*, 76 (11), 1505-1512.

Fukumura, D., Gohongi, T., Kadambi, A., Izumi, Y., Ang, J., Yun, C.O., et al. (2001). Predominant role of endothelial nitric oxide synthase in vascular endothelial

growth factor-induced angiogenesis and vascular permeability. *Proceedings of the National Academy of Sciences of the United States of America,* 98 (5), 2604-2609.

Gabay, C. and Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. *The New England Journal of Medicine*, 340 (6), 448-454.

Gabay, C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research and Therapy,* 8 (Suppl 2): S3.

Gaffen, S.L. and Hajishengallis, G. (2008). A new inflammatory cytokine on the block: Rethinking periodontal disease and the Th1/Th2 paradigm in the context of Th17 cells and IL-17. *Journal of Dental Research*, 87 (9), 817-828.

Galon, J., Fridman, W.H. and Pages, F. (2007). The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. *Cancer Research,* 67 (5), 1883-1886.

Garden, O.A., Pinheiro, D. and Cunningham, F. (2011). All creatures great and small: regulatory T cells in mice, humans, dogs and other domestic animal species. *International Immunopharmacology,* 11 (5), 576-588.

Garlet, G.P., Cardoso, C.R., Mariano, F.S., Claudino, M., de Assis, G.F., Campanelli, A.P., et al. (2010). Regulatory T cells attenuate experimental periodontitis progression in mice. *Journal of Clinical Periodontology,* 37 (7), 591-600.

Geerts, S.O., Nys, M., De M.P., Charpentier, J., Albert, A., Legrand, V. and Rompen, E.H. (2002). Systemic release of endotoxins induced by gentle mastication: association with periodontitis severity. *Journal of Periodontology*, 73 (1), 73-78.

Gemmell, E., McHugh, G.B., Grieco, D.A. and Seymour, G.J. (2001). Co-stimulatory molecules in human periodontal disease tissues. *Journal of Periodontal Research*, 36 (2), 92-100.

Gemmel, E., Yamazaki, K. and Seymour, G.J. (2007). The role of T cells in periodontal disease: homeostasis and autoimmunity. *Periodontology 2000*, 43, 14-40.

George-Gay, B. and Parker, K. (2003). Understanding the complete blood count with differential. *J Perianest Nurs*, 18 (2), 96-114.

Gregory, A.D. and Houghton, A.M. (2011). Tumor-associated neutrophils: new targets for cancer therapy. *Cancer Research*, 71, 2411-2416.

Gerdes, J., Schwab, U., Lemke, H. and Stein, H. (1983). Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *International Journal of Cancer*, 31 (1), 13-20.

Gerdes, J., Lelle, R.J., Pickartz, H., Heidenreich, W., Schwarting, R., Kurtsiefer, L., et al. (1986). Growth fractions in breast cancer determined in situ with the monoclonal antibody Ki-67. *Journal of Clinical Pathology*, 39 (9), 977-980.

Gerner, W., Stadler, M., Hamer, S.E., Klein, D. and Saalmueller, A. (2010). Sensitive detection of Foxp3 expression in bovine lymphocytes by flow cytometry. *Veterinary Immunology and Immunopathology*, 138 (1-2), 154-158.

Gershon, R.K. and Kondo, K. (1970). Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology*, 18 (5), 723-737.

Gieger, T., Rassnick, K., Siegel, S., Proulx, D., Bergman, P., Anderson, C., et al. (2008). Palliation of clinical signs in 48 dogs with nasal carcinomas treated with coarse-fraction radiation therapy. *Journal of American Animal Hospital Association*, 44 (3), 116-123.

Gilreath, J.A., Stenehjem, D.D. and Rodgers, G.M. (2014). Diagnosis and treatment of cancer-related anemia. *American Journal of Hematology*, 89 (2), 203-212.

Glickman, L.T., Gickman, N.W., Moore, G.E., Goldstein, G.S. and Lewis, H.B. (2009). Evaluation of the risk of endocarditis and other cardiovascular events on the basis of the severity of periodontal disease in dogs. *Journal of American Veterinary Medical Association*, 234 (4), 486-494.

Goldman, A.S. and Prabhakar, B.S. (1996). Immunology overview. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; Chapter 1.

Goodman, M., Liu, L., Ward, K., Zhang, J., Almon, L., Su, G., et al. (2009). Invasion characteristics of oral tongue cancer. Frequency of reporting and effect on survival in a population-based study. *Cancer*, 115 (17), 4010-4020.

Goubran, H.A., Stakiw, J., Radosevic, M. and Burnouf, T. (2014). Platelet-cancer interactions. *Seminars in Thrombosis and Hemostasis*, 40 (3), 296-305.

Gratzinger, D., Advani, R., Zhao, S., Talreja, N., Tibshirani, R.J., Shyam, R., et al. (2009). Lymphoma cell VEGFR-2 expression detected by immunohistochemistry predicts poor overall sirvival in diffuse large B cell lymphoma treated with immunochemotherapy (R-CHOP*). British Journal of Haematology*, 148 (2), 235-244.

Graves, D.T. and Cochran, D. (2003). The contribution of interleukin-1 and tumour necrosis factor to periodontal tissue destruction. *Journal of Periodontology*, 74 (3), 391-401.

Grivennikov, S.I., Greten, F.R. and Karin, M. (2010). Immunity, inflammation and cancer. *Cell,* 140 (6), 883-899.

Grossi, C.E., Favre A., Giunta, M. and Corte, G. (1991). T cell differentiation in the thymus. *Cytotechnology*, 1991, 5(1), 113-116.

Guthrie, G.J., Charles, K.A., Roxburgh, C.S., Horgan, P.G., McMillan, D.C. and Clarke, S.J. (2013). The systemic inflammation based beutrophil-lymphocyte ratio:

experience in patients with cancer. *Critical Reviews in Oncology Hematology*, 88 (1), 218-230.

Gürkan, A., Emingil, G., Cinarcik, S. and Berdeli, A. (2006). Gingival crevicular fluid transforming growth factor-beta1 in several forms of periodontal disease. *Archives of Oral Biology*, 51 (10), 906-912.

Gwak, M.S., Choi, S.J., Kim, J.A., Ko, J.S., Kim, T.H., Lee, S.M., et al. (2007). Effects of gender on white blood cell populations and neutrophil-lymphocyte ratio following gastrectomy in patients with stomach cancer. *Journal of Korean Medical Science*, 22: 104-8.

Gwozdz, J.T., Morrison, W.H., Garden, A.S., Weber, R.S., Peters, L.J. and Ang, K.K. (1997). Concomitant boost radiotherapy for squamous carcinoma of the tonsillar fossa. International *Journal of Radiation Oncology Biology Physics*, 39 (1), 127-135.

Haddad, C.R., Guo, L., Clarke, S., Guminski, A., Back, M. and Eade, T. (2015). Neutrophil to lymphocyte ratio in head and neck cancer. *Journal of Medical Imaging and Radiation Oncology*, 59 (4), 514-519.

Hahn, K.A., Ogilvie, G., Rusk, T., Devauchelle, P., Leblanc, A., Legendre, A., et al. (2008). Masitinib is safe and effective for the treatment of canine mast cell tumours. *Journal of Veterinary Internal Medicine*, 22 (6), 1301-1309.

Hall, EJ. (1994). Predictive assays. In: Hall EJ, editor. Radiobiology for the radiobiologist. Philadelphia: Lippincott, p 244-256.

Hall, EJ. (2000). Time, dose and fractionation radiotherapy. In: Hall EJ (ed.): radiobiology for the diagnosis, 5th ed. Philadelphia, PA: Lippincott William & Wilkins, p 397-417.

Halper, J. (2010). Growth factors as active participants in carcinogenesis: a perspective. *Veterinary Pathology,* 47 (1), 77-97.

Hamza, E., Steinbach, F. and Marti, E. (2012). CD4+CD25+ T cells expressing FoxP3 in Icelandic horses affected with insect bite hypersensitivity. *Veterinary Immunology and Immunopathology*, 148 (1-2), 139-144.

Hanahan, D. (1997). Signaling vascular morphogenesis and maintenance. *Science*, 277 (5322), 48-50.

Harari, P.M. Promising new advances in head and neck therapy. (2005). *Annals of Oncology*, 16 (6), 13-19.

Harris, A.L. (2002). Hypoxia-a key regulator in tumour growth. *Nature Reviews Cancer*, 2 (1), 38-47.

Harvey, C.E and Emily, P.P. (1992). Periodontal disease. In: Small Animal Dentistry. Mosby: St. Louis, p 89-144.

Harvey, C.E. (1998). Periodontal disease in dogs: etiopathogenesis, prevalence and significance. *Veterinary Clinics of North America: Small Animal Practice,* 28 (5), 1111-1128.

Hashibe, M., Brennan, P., Benhamou, S., Castellsague, X., Chen, C., Curado, M.P., et al. (2007). Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Journal of the National Cancer Institute*, 99 (10), 777-789.

Havemose-Poulsen, A., Westergaard, J., Stoltze, K., Skjødt, H., Danneskiold-Samsøe, B., Locht, H, Bendtzen, K. and Holmstrup, P. (2006). Periodontal and haematological characteristics associated with aggressive periodontitis, juvenile idiopathic arthritis, and rheumatoid arthritis. *Journal of Periodontology*, 77 (2), 280-288.

Hernandez, E., Lavine, M., Dunton, C.J., Gracely, E. and Parker, J. (1992). Poor prognosis associated with thrombocytosis in patients with cervical cancer. *Cancer*, 69 (12), 2975-2977.

Hernberg, M. (1999). Lymphocyte subsets as prognostic markers for cancer patients receiving immunomodulative therapy. Review. *Medical Oncology*, 16 (3), 145-53.

Hernberg, M., Mattila, P.S., Rissanen, M., Hansson, J., Aamdal, S., Bastholt, L., et al. (2007). The prognostic role of blood lymphocyte subset distribution in patients with resected high-risk primary or regionally metastatic melanoma. *Journal of Immunotherapy*, 30 (7), 773-779.

Herndon, J.E., Green, M.R., Chahinian, A.P., Corson, J.M., Suzuki, Y. and Vogelzang, N.J. (1998). Factors predictive of survival among 337 patients with mesothelioma treated between 1984 and 1994 by the Cancer and Leukemia Group B. *Chest,* 113 (3), 723-731.

Hicklin, D.J. and Ellis, L.M. (2005). Role of the vascular endothelial growth factor pathway in tumour growth and angiogenesis. *Journal of Clinical Oncology*, 23 (5), 1011-1027.

Hirsch, H.Z., Tarkowski, A., Miller, E.J., Gay, S., Koopman, W.J. and Mestecky, J. (1988). Autoimmunity to collagen in adult periodontal disease. *Journal of Oral Pathology & Medicine,* 17 (9–10), 456-459.

Hliniak, A., Gwiazdowska, B., Szutkowski, Z., Kraszewska, E., Kukolowicz, P., Jarzabski, A., et al. (2002). A multicentre randomized/controlled trial of a conventional versus modestly accelerated radiotherapy in the laryngeal cancer: Influence of a 1 week shortening overall time. *Radiotherapy & Oncology,* 62 (1), 1-10.

Hoffmann, T.H. and Gaengler, P. (1996). Clinical and pathomorphological investigation of spontaneously occurring periodontal disease in dogs. *Journal of Small Animal Practice,* 37 (10), 471-479.

Hogquist, K.A., Baldwin, T.A. and Jameson, S.C. (2005). Central tolerance: learning self-control in the thymus. *Nature Reviews Immunology*, 5 (10), 772–782.

Holmqvist, K., Cross, M., Riley, D. and Welsh, M. (2003). The Shb adaptor protein causes Src-dependent cell spreading and activation of focal adhesion kinase in murine brain endothelial cells. *Cell Signal,* 15 (2), 171-179.

Hori, S., Takahashi, T. and Sakaguchi, S. (2003). Control of autoimmunity by naturally arising regulatory CD4+ T cells. *Advances in Immunology*, 81, 331-371.

Horiuchi, Y., Tominaga, M., Ichikawa, M., Yamashita, M., Jikumaru, Y., Nariai, Y., et al. (2009). Increase of regulatory T cells in the peripheral blood of dogs with metastatic tumours. *Microbiology and Immunology*, 53 (8), 468-474.

Horiuchi, Y., Tominaga, M., Ichicava, M., Yamashita, M., Okano, K., Jikumaru, Y., et al. (2010). Relationship between regulatory and type 1 T cells in dogs with oral malignant melanoma. *Microbiology and Immunology*, 54 (3), 152-159.

Horiot, J.C., Le Fur, R., N'Guyen, T., Chenal, C., Schraub, S., Alfonsi, S., et al. (1992). Hyperfractionation versus conventional fractionation in oropharyngeal carcinoma: Final analysis of a randomized trial of the EORTC cooperative group of radiotherapy. *Radiotherapy & Oncology,* 25 (4), 231-241.

Horiot, J.C., Bontemps, P., van den Bogaert, W., Le Fur, R., van den Weijngaert, D., Bolla M., et al. (1997). Accelerated fractionation (AF) compared to conventional fractionation (CF) improves loco-regional control in the radiotherapy of advanced head and neck cancers: Results of the EORTC 22851 randomized trial. *Radiotherapy & Oncology*, 44 (2), 111-121.

Houri-Haddad, Y., Wilensky, A. and Shapira, L. (2007). T-cell phenotype as a risk factor for periodontal disease. *Periodontology 2000*, 45, 67-75.

Hurter B. and Bush N.J. (2007). Cancer-related anemia: clinical review and management update. *Clinical Journal of Oncology Nursing*, 11 (3), 349-359.

Hutter, J.W., van der Velden, U., Varoufaki, A., Huffels, R.A., Hoek, F.J. and Loos B.G. (2001). Lower numbers of erythrocytes and lower levels of haemoglobin in periodontitis patients compared to control subjects. *Journal of Clinical Periodontology*, 28 (10), 930-936.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., et al. (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *The New England Journal of Medicine*, 350 (23), 2335-2342.

Indumathy, P., Arun, K.V., Kuman, T.S.S., Raja, A. and Sai Prashanth, P. (2012). Comparison of systemic levels of regulatory T cells in periodontal health and disease. *Journal of Dr. University of Health Sciences,* 1 (3), 156-162.

Innocenti, M., Frittoli, E., Ponzanelli, I., Falck, J.R., Brachmann, S.M., Di Fiore, P.P. and Scita, G. (2003). Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. *Biology of the Cell,* 160 (1), 17-23.

Ito H., Honda T., Domon H., Oda T., Okui T., Amanuma R., et al. (2005). Gene expression analysis of the CD4+ T-cell clones derived from gingival tissues of periodontitis patients. *Microbiology and Immunology*, 20 (6), 382-386.

Itoh, M., Takashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F. and Sakaguchi, S. (1999). Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *Journal of Immunology*, 162 (9), 5317-5326.

Jackson, S.M., Weir, L.M., Hay, J.H., Tsang, V.H and Durhamn, J.S. (1997). A randomised trial of accelerated versus conventional radiotherapy in head and neck cancer. *Radiotherapy & Oncology,* 43 (1), 39-46.

Janeway, C.A. Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol,* 54, 1-13.

Janeway, C.A. Jr. (1992). The immune system evolved to discriminate infectious non-self from non-infectious self. *Immunology Today*, 13 (1), 11-16.

Janeway, C.A. Jr. and Medzhitov, R. (2002). Innate immune recognition. *Annual Review of Immunology,* 20, 197-216.

Joshi, A., Vahlenkamp, T.W., Garg, H., Tompkins, W.A. and Tompkins, M.B. (2004). Preferential replication of FIV in activated CD4+CD25+ T cells independent of cellular proliferation. *Virology,* 321 (2), 307-322.

Jotwani, R., Palucka, A.K., Al-Quotub, M., Nouri-Shirazi, M., Kim, J., Bell, D., et al. (2001). Mature dendritic cells infiltrate the T cell-rich region of oral mucosa in chronic periodontitis: in situ, in vivo and in vitro studies. *Journal of Immunology*, 167 (8), 4693-4700.

Junginger, J., Schwittlick, U., Lemensieck, F., Nolte, I. and Hewicke-Trautwein, M. (2012). Immunohistochemical investigation of Foxp3 expression in the intestine in healthy and diseased dogs. Veterinary Research, 43 (1), 23.

Kalburgi, N.B., Muley, A., Shivaprasad, B.M., and Koregol, A.C. (2013). Expression Profile of IL-35 mRNA in gingiva of chronic periodontitis and aggressive periodontitis patients: A semiquantitative RT-PCR study. *Disease Markers*, 35 (6), 819-823.

Kaplan, K.L., Broekman, M.J., Chemoff, A., Lesznik, G.R. and Drillings, M. (1979). Platelet alpha-granule proteins: studies on release and subcellular localization. *Blood,* 53 (4), 604-618.

Kaser, T., Gerner, W., Hammer, S.E., Patzl ,M. and Saalmueller, A. (2008). Detection of Foxp3 protein expression in porcine T lymphocytes. *Veterinary Immunology and Immunopathology*, 125 (1-2), 92-101.

Kasuga, I., Makino, S., Kiyokawa, H., Katoh, H., Ebihara, Y. and Ohyashiki, K. (2001). Tumour related leukocytosis is linked with poor prognosis in patients with lung carcinoma. *Cancer*, 92 (9), 2399-2405.

Katz, J., Goultschin, J., Benoliel, R. and Schlesinger, M. (1988). Peripheral T lymphocyte subsets in rapidly progressive periodontitis. *Journal of Clinical Periodontology*, 15 (4), 266-268.

Kee, Y.Y., Choi, C.H., Kim, H.J., Kim, T.J., Lee, J.W., Lee, J.H., et al. (2012). Pretreatment neutrophil/lymphocyte ratio as a prognostic factor in cervical carcinoma. *Anticancer Research,* 32 (4): 1555-1562.

Kennedy, A.S., Raleigh, J.A., Perez, G.M., Calkins, D.P., Thrall, D.E., Novotny, D.B. and Varia, M.A. (1997). Proliferation and hypoxia in human squamous cell carcinoma of the cervix: first report of combined immunohistochemical assays. *International Journal of Radiation Oncology Biology Physics*, 37 (4), 897-905.

Keppel, K.E., Campbell, K.L., Zuckerman, F.A., Greeley, E.A., Schaeffer, D.J. and Husmann, R.J. (2008). Quantitation of canine regulatory T cell populations, serum interleukin-10 and allergen-specific IgE concentrations in healthy control dogs and canine atopic dermatitis patients receiving allergen-specific immunotherapy. *Veterinary Immunology and Immunopathology,* 123, 337-344.

Khalaf, H. and Benghtsson, H. (2012). Altered T-Cell responses by the periodontal pathogen *Porphyromonas gingivalis*. *Plos One*, 7 (9), e45192.

Kilincalp S., Ekiz, F., Başar, O., Ayte, M.R., Coban, S., Yılmaz, B., Altınbaş, A., et al. (2014). Platelet volume could be possible biomarker in early diagnosis and monitoring of gastric cancer. *Platelets*, 25 (8), 592-594.

Kim, I.Y., You, S.H. and Kim, Y.W. (2014). Neutrophil-lymphocyte ratio predicts pathologic tumour response and survival after preoperative chemoradiation for rectal cancer. *BMC Surgery,* 14, 94.

Kim, B., Suvas, S., Sarangi, P.P., Lee, S., Reisfeld, R.A. and Rouse, B.T. (2006). Vascular endothelial growth factor receptor 2 – based DNA immunization delays development of herpetic stromal keratitis by antiangiogenic effects. *Journal of Immunology*, 177 (6), 4122-4131.

Kim, Y.C., Ko, Y., Hong, S.D., Kim, K.Y., Lee, Y.H., Chae, C. and Choi, Y. (2010). Presence of Porphyromonas gingivalis and plasma cell dominance in gingival tissues with periodontitis. *Oral Diseases*, 16 (4), 375-381.

Kinane, D.F., Johnston, R.A. and Evans, C.W. (1989). Depressed helper-to-suppressor T-cell ratios in early-onset forms of periodontal disease. *Journal of Periodontal Research,* 24 (3), 161-164.

Kinane, D.F. and Lappin, D.F. (2001). Clinical, pathological and immunological aspects of periodontal disease. *Acta Odontologica Scandinavica*, 59 (3), 154-160.

Kirby, A.M., A'Hern, R.P., D'Ambrosio, C., Tanay, M., Syrigos, K.N., Rogers, S.J., et al. (2006). Gefitinib (ZD1839, Iressa™) as palliative treatment in recurrent or metastatic head and neck cancer. *British Journal of Cancer*, 94 (5), 631-636.

Kleiter, M., Malarkey, D.E., Ruslander, D.E. and Thrall, D.E. (2004). Expression of cyclooxygenase-2 in canine epithelial nasal tumours. *Veterinary Radiology & Ultrasound*, 45 (3), 255-260.

Klinger, M.H. and Jelkmann, W. Role of blood platelets in infection and inflammation. *Journal of Interferon & Cytokine Research,* 22 (9), 913-922.

Koch, S., Tugues, S., Li X., Gualandi, L. and Claesson-Welsh, L. (2011). Signal transduction by vascular endothelial growth factor receptors. *Journal of Biochemistry*, 437 (2), 169-183.

König, J., Holtfreter, B. and Kocher, T. (2010). Periodontal health in Europe: future trends based on treatment needs and the provision of periodontal services--position paper 1. European Journal of Dental Education, 14 (1): 4-24.

Kono, S.A., Heasley, L.E., Doebele, R.C. and Camidge, D.R. (2012). Adding to the mix: fibroblast growth factor and platelet derived factor receptor pathways as targets in non-small cell lung cancer. *Current Cancer Drug Targets*, 12 (2), 107-123.

Kramer, R.H., Shen, X. and Zhou. H. (2005). Tumour cell invasion and survival in head and neck cancer. *Cancer and Metastasis Reviews*, 24 (1), 35-45.

Kumar, V. and Sharma, A. (2010). Neutrophils: Cinderella of innate immune system. *International Immunopharmacology*, 10 (11), 1325-1334.

Kusumanto, Y.H., Dam, W.A., Hospers, G.A., Meijer, C. and Mulder, N.H. (2003). Platelets and granulocytes, in particular the neutrophils, form important compartments for circulating vascular endothelial growth factor. *Angiogenesis*, 6 (4), 283-287.

Kwon, H.C., Kim, S.H., Oh, S.Y., Lee, S., Lee, J.H., Choi, H.J., et al. (2012). Clinical significance of preoperative neutrophil to lymphocyte versus platelet-lymphocyte ratio in patients with operable colorectal cancer. *Biomarkers,* 17 (3), 216-222.

Kyzas, P.A., Stefanou, D., Batistatou, A. and Agnantis, N.J. (2005). Potential autocrine function of vascular endothelial growth factor in head and neck cancer via vascular endothelial growth factor receptor 2. *Modern Pathology,* 18 (4), 485-494.

LaDue, T.A., Dodge, R., Page, R.L., Price, G.S., Hauck, M.L. and Thrall, D.E. (1999). Factors influencing survival after radiotherapy of nasal tumours in 130 dogs. *Veterinary Radiology & Ultrasound,* 40 (3), 312-317.

LaDue, T., Klein, M.K. (2001). Toxicity criteria of the veterinary radiation oncology group. Vet Radiol and Ultrasound, 42 (5), 475-476.

Lalla, R.V., Boisoneau, D.S., Spiro, J.D. and Kreuztzer, D.L. (2003). Expression of vascular endothelial growth factor receptors on tumour cells in head and neck squamous cell carcinoma. *Archives of Otolaryngology - Head and Neck Surgery*, 129 (8), 882-888.

Lana, S.E., Dernell, W.S., LaRue, S.M., Lafferty, M.J., Douple, E.B., Brekke, J.H. and Withrow, S.J. (1997). Slow release of cisplatin combined with radiation for the treatment of canine nasal tumours. *Veterinary Radiology Ultrasound*, 38 (6), 474-478.

Lankford, S., Petty, C., Lavoy, A., Recklong, S., Tompkins, W. and Dean, G.A. (2008). Cloning of feline FOXP3 and detection of expression in CD4+CD25+ regulatory T cells. *Veterinary Immunology and Immunopathology*, 122 (1-2), 159-166.

LeBlanc, A.K., LaDue, T.A., Turrel, J.M., and Klein, M.K. (2004). Unexpected toxicity following use of gemcitabine as a radiosensitizer in head and neck carcinomas: a veterinary radiation therapy oncology group pilot study. *Veterinary Radiology & Ultrasound*, 45 (5), 466-470.

Laird, B.Y., Mcmillan, D.C., Fayers, P., Fearon, K., Kaasa, S., Fallon, M.T. and Klepstad, P. (2013). The systemic inflammatory response and its relationship to pain and other symptoms in advanced cancer. *The Oncologist*, 18 (9), 1050-1055.

Lee, S.L., Rouhi, P., Dahl Jensen, L., Zhang, D., Ji, H., Hauptmann, G., et al. (2009). Hypoxia-induced pathological angiogenesis mediates tumor cell dissemination, invasion, and metastasis in a zebrafish tumor model. *Proceedings of the National Academy of Sciences of the United States of America,* 106 (46), 19485-19490.

Lee, Y., Kim, S.H., Han, J.Y., Kim, H.T., Yun, T. and Lee, J.S. (2012). Early neutrophil to lymphocyte ratio reduction as a surrogate marker of prognosis in never smokers with advanced lung adenocarcinoma receiving gefitinib or standard chemotherapy as first-line therapy. *Journal of Cancer Research and Clinical Oncology*, 138 (12), 2009-2016.

Leek, R.D., Landers, R.J., Harris, L. and Lewis, C.E. (1999). Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. *British Journal of Cancer,* 79 (5-6), 991-995.

Lerman, M.A., Larkin, J., Cozzo, C., Jordan, M.S. and Caton, A.J. (2004). CD4+CD25+ regulatory T cell repertoire formation in response to varying expression of a neo-selfantigen. *Journal of Immunology***,** 173 (1), 236-244.

Levin, J. and Conley, C. (1964). Thrombocytosis associated with malignant disease. *Archives of Internal Medicine,* 114, 497-500.

Li, C., Shintani, S., Terakado, N., Klosek, S.K., Ishikawa, T., Nakashiro, K. and Hamakawa, H. (2005). Microvessel density and expression of vascular growth factor and platelet derived endothelial growth factor in oral squamous cell carcinomas. *International Journal of Oral and Maxillofacial Surgery,* 34 (5), 559-565.

Lin, S.C., Chen, K.H., Lin, C.H., Kuo, C.C., Ling, Q.D. and Chan, C.H. (2007). The quantitative analysis of peripheral blood FOXP3-expressing T cells in systemic lupus erythematosus and rheumatoid arthritis patients. *European Journal of Clinical Investigation,* 37 (12), 987-996.

Liptak, J.M. and Withrow, S.J. (2007). Cancer of the gastrointestinal tract. Section A: Oral tumors. In: Withrow & MacEwen's Small Animal Clinical Oncology, 4th Edit., Withrow SJ, Vail DM, Eds. Saunders Elsevier, St Louis, p 455-475.

Liu, Y., Cox, S.R., Morita, T. and Kourembanas, S. (1995). Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circulation Research*, 77 (3), 638-643.

London, C., Mathie, T., Stingle, N., Clifford, C., Haney, S., Klein, M.K., et al. (2012). Preliminary evidence for biologic activity of toceranib phosphate (Palladia(®)) in solid tumours. *Veterinary and Comparative Oncology*, 10 (3), 194-205.

Loos, B.G. (2005). Systemic markers of inflammation in periodontitis. *Journal of Periodontology,* 76 (11 Suppl), 2106-2115.

Lopes, J.E., Torgerson, T.R., Schubert, L.A., Anover, S.D., Ocheltree, E.L., Ochs, H.D. and Ziegler, S.F. (2006). Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor. *Journal of Immunology*, 177 (5), 3133-3142.

Lund, F.E., Garvy, B.A., Randall, T.D. and Harris, D.P. (2005). Regulatory roles for cytokine producing B cells in infection and autoimmune disease. *Curr Dir Autoimmu*, 8: 25-54.

Macchiarini, P., Fontanini, G., Hardin, M.J., Squartini, F. and Angeletti, C.A. (1992). Relation of neovascularisation to metastasis of non-small-cell lung cancer. *Lancet,* 340 (8812), 145-146.

MacEwen, E.G., Withrow, S.J. and Patnaik, AK. (1977). Nasal tumours in the dog: retrospective evaluation of diagnosis, prognosis and treatment. *Journal of American Veterinary Medical Association*, 170 (1), 45-48.

MacKay, I.R. (2001). Tolerance and autoimmunity. *Western Journal of Medicine*, 174 (2), 118-123.

Madewell, B.R., Priester, W.A., Gillete, E.L. and Snyder, S.P. (1976). Neoplasms of the nasal passages and paranasal sinuses in domesticated animals as reported by 13 veterinary colleagues. *American Journal of Veterinary Research*, 37 (7), 851-856.

Majlessi, L., Lo-Man, R. and Leclerc, C. (2008). Regulatory B and T cells in infections. *Microbes and Infection*, 10 (9), 1030-1035.

Malek, T.R. and Bayer, A. (2004). Tolerance, not immunity, crucially depends on IL-2. *Nature Reviews Immunology,* 4 (9), 665-674.

Maloy, K.J. and Powrie, F. (2001). Regulatory T cells in the control of the immune pathology. *Nature Immunology*, 2 (9), 816-22.

Mangalpally, K.K., Siqueiros-Garcia, A., Vaduganathan, M., Dong, J.F., Kleiman, N.S. and Guthikonda, S. (2010). Platelet activation patterns in platelet size subpopulations: differential responses to aspirin in vitro. *Journal of Thrombosis and Thrombolysis*, 30 (3), 251-262.

Manigold, T., Shin, E.C., Mizukoshi, E., Mihalik, K., Murthy, K.K., Rice, C.M., et al. (2006). Foxp3+CD4+CD25+ T cells control virus-specific memory T cells in chimpanzees that recovered from hepatitis C. *Blood*, 107 (11), 4424-4432.

Manti, F., Kornman, K. and Goldschneider, I. (1984). Effects of an immunomodulating agent on peripheral blood lymphocytes and subgingival microflora in ligature-induced periodontitis. *Infection and Immunity*, 45 (1), 172-179.

Mantovani, A., Allavena, P., Sica, A. and Ballkwill, F. (2008). Cancer related inflammation. *Nature*, 454 (7203): 436-444.

Marcu, L.G. (2013). Improving therapeutic ratio in head and neck cancer with adjuvant and cisplatin-based treatments. *BioMed Research International,* 2013, 817279, 9 pages.

Martin, J.F., Plumb, J., Kilbey, R.S. and Kishk, Y.T. (1983). Changes in volume and density of platelets in myocardial infarction. *British Medical Journal (Clinical Research Ed.),* 287 (6390), 456-459.

Martini, V. Clinical pathological features of canine hematopoietic neoplasms assessed via flow cytometry. University of Padua. Doctoral thesis. Corso di dottorato, 2013, pp 10.

Mauldin, G.N., Matus, R.E., Patnaik, A.K., Bond, B.R. and Mooney, S.C. (1988). Efficacy and toxicity of doxorubicin and cyclophosphamide used in the treatment of

selected malignant tumours in 23 cats. *Journal of Veterinary Internal Medicine*, 2 (2), 60-65.

McEntee, M.C. (2012). Clinical behaviour of nonodontogenic tumours. In: Verstraete FJM, Lommer MJ, Bezuidenhout AJ, editors. Oral and Maxillofacial Surgery in Dogs and Cats. Edinburgh: Saunders/Elsevier; 2012. P 387-402.

McEntee, M.C., Page, R.L., Heidner, G.L., Cline, J.M. and Thrall, D.E. (1991). A retrospective study of 27 dogs with intranasal neoplasms treated with cobalt radiation. *Veterinary Radiology & Ultrasound,* 32:135-139.

McKee, L.C. (1985). Excess leucocytosis (leukemoid reactions) associated with malignant diseases. *Southern Medical Journal*, 78 (12), 1475-1482.

McMillan, D.C. (2009). Systemic inflammation, nutritional status and survival in patients with cancer. *Current opinion in clinical nutrition and metabolic care*, 12 (3), 223-226.

Mcneilly, T.N., McIntyre, J., Frew, D., Griffiths, D.J., Wattegedera, S.R., Van Den Broeck, A. and Huntley, F. (2010). Infestation of sheep with Psoroptes ovis, the sheep scab mite, results in recruitment of Foxp3(+) T cells into the dermis. *Parasite Immunology*, 32 (5), 361-369.

Mellanby, R.J., Stevenson, R.K., Herrtage, M.E., White, R.A. and Dobson, J.M. (2002). Long-term outcome of 56 dogs with nasal tumours treated with four doses of radiation at intervals of seven days. *Veterinary Record*, 151 (9), 253-257.

Mendenhall, W.M., Amdur, R.J., Stringer, S.P., Villaret, D.B. and Cassisi, N.J. (2000). Radiation therapy for squamous cell carcinoma of the tonsillar region: A preferred alternative to surgery? *Journal of Clinical Oncology and Research*, 18 (11), 2219-2225.

Meng, H.X. and Zheng, L.F. (1989). T cells and T-cell subsets in periodontal diseases. *Journal of Periodontal Research*, 24 (2), 121-126.

Mexas, A.M., Fogle, J.E., Tompkins, W.A. and Tompkins, M.B. (2008). CD4+CD25+ regulatory T cells are infected and activated during acute FIV infection. *Veterinary Immunology and Immunopathology,* 126 (3-4), 263-272.

Millanta, F., Silvestri, G., Vaselli, C., Citi, S., Pisani, G., Lorenzi, D. and Poli, A. (2006). The role of vascular endothelial growth factor and its receptor Flk-1/KDR in promoting tumour angiogenesis in feline and canine mammary carcinomas: A preliminary study of autocrine and paracrine loops. *Research in Veterinary Science*, 81, 350-357.

Million, R.R., Parsons, J.T. and Cassisi, N.J. (1985). Twice-a-day irradiation technique for squamous cell carcinomas of the head and neck. *Cancer*, 55(9 suppl), 2096-2099.

Mills, K.H. (2004). Regulatory T cells: friend or foe in immunity to infection? *Nature Reviews Immunology,* 4 (11), 841-855.

Minchenko, A., Salceda, S., Bauer, T. and Caro, J. (1994). Hypoxia regulatory elements of the human vascular endothelial growth factor gene. *Molecular Cell Biology Research Communications,* 40 (1), 35-39.

Mitra, S., Alnabulsi, A., Secombes, C.J. and Bird, S. (2010). Identification and characterization of the transcription factors involved in T-cell development, t-bet, stat6 and foxp3, within the zebrafish, Danio rerio. *Federation of European Biochemical Societies Journal*, 277 (1), 128-147.

Mizuno, T., Suzuki, R., Umeki, S. and Okida, M. (2009). Crossreactivity of antibodies to canine CD25 and Foxp3 and identification of canine CD4+CD25+Foxp3+ cells in canine peripheral blood. *Journal of Veterinary Medical Science*, 71 (12), 1561-1568.

Modiano, J.F., Ritt, M.G. and Wojcieszyn, J. (1999). The molecular basis of canine melanoma: pathogenesis and trends in diagnosis and therapy. *Journal of Veterinary Internal Medicine*, 13 (3), 163-174.

Monreal, M., Fernandez-Llamazares, J., Piñol, M., Julian, J.F., Broggi, M., Escola, D. and Abad, A. (1998). Platelet count and survival in patients with colorectal cancer - a preliminary study. *Journal of Thrombosis and Haemostasis*, 79 (5), 916-918.

Monteiro, A.M., Jardini, M.A., Alves, S., Giampaoli, V., Aubin, E.C., Figueiredo Neto, A.M. and Gidlund, M. (2009). Cardiovascular disease parameters in periodontitis. *Journal of Periodontology,* 80 (3), 378–388.

Mori, G., Brunetti, G., Colucci, S., Ciccolella, F., Coricciati, M., Pignataro, P., et al. (2007). Alteration of activity and survival of osteoblasts obtained from human periodontitis patients: role of TRAIL. *Journal of Biological Regulators & Homeostatic Agents*, 21 (3-4), 105-114.

Moriyama, M., Kumagi, S., Kaeashiri, S., Kojima, K., Kakihara, K. and Yamamoto, E. (1997). Immunohistochemical study of tumour angiogenesis in oral squamous cell carcinoma. *Oral Oncology,* 33 (5), 369-374.

Morris, J.S., Dunn, K.J., Dobson, J.M. and White, R.A.S. (1994). Effects of radiotherapy alone and surgery and radiotherapy on survival of dogs with nasal tumors. *Journal of Small Animal Practice,* 35, 567-573.

Mosmann, T.R. and Coffman, R.L. (1989). Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology,* 7, 145-173.

Mosmann, T.R. and Sad, S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology Today,* 17 (3), 138-146.

Motomura, T., Shirabe, K., Mano, Y., Muto, J., Toshima, T., Umemoto, Y., et al. (2013). Neutrophil-lymphocyte ratio reflects hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment. *Journal of Hepatology*, 58 (1), 58–64.

Mottet, C., Uhlig, H.H. and Powrie, F. (2003). Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *Journal of Immunology*, 170, (8), 3939-3943.

Moutsopoulos, N.M. and Madianos, P.N. (2006). Low-grade inflammation in chronic infectious diseases: paradigm of periodontal infections. *Annals of the New York Academy of Sciences,* 1088, 251-264.

Mukhopadhyay, D., Tsiokas, L. and Sukhatme, V.P. (1995). Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. *Cancer Research,* 55 (24), 6161-6165.

Murphy, K. (2012). Janeway's Immunobiology: 8th ed. Chapter 15: Garland Science. pp. 611–668.

Mutz, M., Boudreaux, B., Kearney, M., Stroda, K., Gaunt, S. and Shiomitsu, K. (2015). Prognostic value of baseline absolute lymphocyte concentration and neutrophil/lymphocyte ratio in dogs with newly diagnosed multi-centric lymphoma. *Veterinary and Comparative Oncology*, 13 (4), 337-347.

Nadeau, M.E., Kitchell, B.E., Rooks, R.L. and LaRue, S.M. (2004). Cobalt radiation with or without low-dose cisplatin for treatment of canine naso-sinus carcinomas. *Veterinary Radiology and Ultrasound,* 45 (4), 362-367.

Nair, S., Faizuddin, M. and Dharmapalan, J. (2014). Role of autoimmune responses in periodontal disease. *Hindawi Publishing Corporation Autoimmune Diseases*, 596824, 7 pages.

Nakajima, T., Ueki-Maruyama, K., Oda, T., Ohsawa, Y., Ito, H., Seymour, G.J. and Yamazaki, K. (2005). Regulatory T-cells infiltrate periodontal disease tissues. *Journal of Dental Research*, 84 (7), 639-643.

Nakamura, K., Kitani, A. and Strober, W. (2001). Cell contact-dependent immunosuppression by CD4+CD25+ regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *Journal of Experimental Medicine*, 194 (5), 629-644.

Negasawa, T., Nitta, H., Watanabe, H. and Ishikawa, I. (1995). Reduced CD8+ peripheral blood T lymphocytes in rapidly progressive periodontitis. *Archives of Oral Biology,* 40 (7), 605-608.

Nemec, A, Petelin, M, Pavlica, Z. (2013). Systemic inflammatory host response to periodontopathogenic bacteria in the oral cavity: from experimental to clinical studies. In: Mendez-Vilas A. (ed.). Microbial pathogens and strategies for combating

them: science, technology and education, (Microbiology book series, 4). *Badajoz: Formatex Research Center*, pp 1926-1933.

Neuchrist, C., Erlivic, B.M., Handisurya, A., Steiner, G.E., Rochweill, P., Gedlicka, C. and Burian, M. (2001). Vascular endothelial growth factor receptor 2 (VEGFR-2) expression in squamous cell carcinomas of the head and neck. *Laryngoscope,* 111 (10), 1834-1841.

Neufeld, G., Cohen, T., Gengrinovitch, S. and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *Federation of American Societies for Experimental Biology Journal*, 3 (1), 9-22.

Nguyen, S.M., Thamm, D.H., Vail, D.M. and London, C.A. (2015). Response evaluation criteria for solid tumours in dogs (v1.0): a Veterinary Cooperative Oncology Group (VCOG) consenus document. *Veterinary and Comparative Oncology*, 13 (3), 176-183.

Nguyen, D., Diamond, L.W. and Braylan, R.C. (2007). A visual approach to data analysis and interpretation. In: Flow cytometry in hematopathology 2nd edition, Totowa, NJ, Humana Press. P 1-48.

Nico, B., Benagiano, V., Mangieri, D., Maruotti, N., Vacca, A. and Ribatti, D. (2008). Evaluation of microvascular density in tumours: pro et contra. *Histology and Histopathology*, 23 (5), 601-607.

Nichols, A.C., Whelan, F., Basmaji, J., Dhaliwal, S., Dowthwaite, S., Chapeskie, C., et al. (2012). Ki-67 expression predicts radiotherapy failure in early glottic cancer. *Journal of Otolaryngology-Head & Neck Surgery,* 41 (2): 124-130.

Nilson, I., Roiny, C., Wu, Y., Pytowski, B., Hicklin, D., Alitalo, K., Claesson-Welsh, L. and Wennstrom, S. (2004). Vascular endothelial growth factor receptor 3 in hypoxia induced vascular development. *Federation of American Societies for Experimental Biology Journal,* 18 (13), 1507-1515.

Noack, B., Genco, R.J., Trevisan, M., Grossi, S., Zambob, J.J and De Nardin, E. (2001). Periodontal infections contribute to elevated systemic C-reactive protein level. *Journal of Periodontology*, 72 (9), 1221-1227.

Northrup, N.C., Etue, S.M., Ruslander, D.M., Rassnick, K.M., Hutto, D.L., Bengtson, A., et al. (2001). Retrospective study of orthovoltage radiation therapy for nasal tumours in 42 dogs. *Journal of Veterinary Internal Medicine*, 15 (3), 183-189.

Nouri-Aria, K.T. and Durham, S.R. (2008). Regulatory T cells and allergic disease. *Inflammation & Allergy - Drug Targets*, 7 (4), 237-252.

Offenbacher, S., Barros, S.P. and Beck, J.D. (2008). Rethinking periodontal inflammation. *Journal of Periodontology*, 79 (8), 1577-1584.

Ogilvie, G.K., Moore, A.S., Obradovich, J.E., Elmslie, R.E., Vail, D.M., Straw, R.C., et al. (1993). Toxicoses and efficacy associated with administration of mitoxantrone

to cats with malignant tumours. *Journal of American Veterinary Medical Association*, 202 (11), 1839-1844.

Okada, H., Kassai, Y. and Kida, T. (1984). T lymphocyte subsets in the inflamed gingiva of human adult periodontitis. *Journal of Periodontal Research*, 19 (6), 595-598.

Okui, T., Ito, H., Honda, T., Amanuma, R., Yoshie, H. and Yamazaki, K. (2008). Characterization of CD4+FOXP3+ T-cell clones established from chronic inflammatory lesions. *Oral Microbiology and Immunology*, 23 (1), 49-54.

Okui, T, Aoki, Y, Ito, H, Honda, T, Yamazaki, K. (2012). The Presence of IL-17+/FOXP3+ Double-positive Cells in Periodontitis. *Journal of Dental Research*, 91 (6), 574-579.

Olsson, A.K., Dimberg, A., Kreuger, J. and Claesson-Welsh, L. (2006). VEGF receptor signalling in control of vascular function. *Nature Reviews Molecular and Cellular Biology*, 7 (5), 359-371.

O'Neill, K., Guth, A., Biller, B., Elmslie, R. and Dow, S. (2009). Changes in regulatory T cells in dogs with cancer and associations with tumor type. *Journal of Veterinary Internal Medicine*, 23 (4), 875-881.

Orbak R., Erciyas K. and Kaya H. (2003). Flow cytometric analysis of T-lymphocyte subsets after different treatment methods in smokers and non-smokers with chronic periodontitis. *International Dental Journal*, 53 (3), 159-164.

Orre, M. and Rogers, P.A.W. (1999). VEGF, VEGFR-1, VEGFR-2, microvessel density and endothelial cell proliferation in tumours of the ovary. *International Journal of Cancer (Pred. Oncol.)*, 84, 101-108.

Ouaguia, L., Mrizak, D., Renaud, S., Moralès, O. and Delhem, N. (2014). Control of the inflammatory response mechanisms mediated by natural and induced regulatory T-cells in HCV-, HTLV-1-, and EBV-associated cancers. *Mediators of Inflammation*, 2014; 564296.

Overgaard, J., Hansen, H.S., Specht, L., Overgaard, M., Grau, C., Anderson, E., et al. (2003). Five compared with six fractions per week of conventional radiotherapy of squamous-cell carcinoma of head and neck: DAHANCA 6 and 7 randomised controlled trial. *Lancet*, 362 (9388), 933-940.

Ozao-Choy, J., Ma, G., Kao, J., Wang, G.X., Meseck, M., Sung, M., et al. (2009). The novel role of tyrosine kinase inhibitor in the reversal of the immune suppression and modulation of tumour microenvironment for immune-based cancer therapies. *Cancer Research,* 69 (6), 2514-2522.

Owen, L.N. (1980). TNM classification of tumours in domestic animals. Geneva, World Health Organization.

Papetti, M. and Herman, I.M. (2002). Mechanisms of normal and tumour-derived angiogenesis. *American Journal of Physiology: Cell Physiology*, 282 (5), 947-970.

Pascual, V., Liu, Y.J., Magalski, A., de Boutellier, O., Banchereau, J. and Capra, J.D. (1994). Analysis of somatic mutation in five B cell subsets of human tonsil. *Journal of Experimental Medicine,*180 (1), 329-339.

Passoja, A., Puijola, I., Knuuttila, M., Niemelä, O., Karttunen, R., Raunio T. and Tervonen T. (2010). Serum levels of interleukin-10 and tumour necrosis factor-alpha in chronic periodontitis. *Journal of Clinical Periodontology*, 37 (10), 881-887.

Patnaik, A.K., Lieberman, P.H., Erlandson, R.A. and Liu, S.K. (1984). Canine sinonasal skeletal neoplasms: chondorsarcomas and osteosarcomas. *Veterinary Pathology*, 21, 475-482.

Patnaik, A.K. (1989). Canine sinonasal neoplasms: clinicopathological study of 258 cases. *Journal of American Animal Hospital Association*, 25, 103-114.

Pavlica, Z., Petelin, M., Juntes, P., Eržen, D., Crossley, D.A. and Skalerič, U. (2008). Periodontal disease burden and pathological changes in organs of dogs. *Journal of Veterinary Dentistry*, 25 (2), 97-105.

Pejčić, A, Kesic, L., Pešic, Z., Mirkovic, D. and Stojanovic, M. (2011). White blood cell count in chronic periodontitis. Acta Clinica Croatica, 50, 159-167.

Peña, L.L., Nieto, A.I., Pérez-Alenza, D., Cuesta, P. and Castaño, M. (1998). Immunohistochemical detection of Ki-67 and PCNA in canine mammary tumours: relationship to clinical and pathologic variables. *Journal of Veterinary Diagnostic Investigation,* 10 (3), 237-246.

Peng, Y., Laoua, Y., Li, M.O., Green, E.A. and Flavell, R.A. (2004). TGF-β regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proceedings of the National Academy of Sciences of the United States of America,* 101 (13), 4572-4527.

Peterson, A.C., Swiger, S., Stadler, W.M., Medved, M., Karczmar, G. and Gajewski, T.F. (2004). Phase II study of the Flk-1 tyrosine kinase inhibitor SU5416 in advanced melanoma. *Clinical Cancer Research,* 10 (12 Pt 1), 4048-4054.

Petznek, H., Kleiter, M., Tichy, A., Huchs-Baumgartinger, A. and Hohenaadl, C. (2014). Murine xenograft model demonstrates significant radiosensitising effect of liposomal doxorubicin in a combination therapy for feline injection site sarcoma. *Research in Veterinary Science*, 97 (2), 386-390.

Pfifer, J.D., Wick, M., Roberts, R.L., Findley, K., Normak, S.J. and Harding, C.V. (1993). Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature,* 361 (6410), 359-362.

Piccirillo, C.A. and Thornton, A.M. (2004). Cornerstone of peripheral tolerance: naturally occurring CD4+CD25+ regulatory T cells. *Trends in Immunology*, 25 (7), 374–380.

Piccirillo, C.A. (2008). Regulatory T cells in health and disease. *Cytokine*, 43 (3), 395-401.

Pich, A., Chiusa, L. and Navone, R. (2004). Prognostic relevance of cell proliferation in head and neck tumours. *Annals of Oncology*, 15 (9), 1319-1329.

Pignon, J.P., Bourhis, J., Domenge, C. and Designe, L. (2000). Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: Three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-analysis of chemotherapy on head and neck cancer. *Lancet,* 355 (9208), 949-955.

Pihlstrom, B.L., Michalowicz, B.S. and Johnson, N.W. (2005). Periodontal diseases. *Lancet*, 366 (9499), 1809-1820.

Pillay, J., Kamp, V.M., van Hoffen, E., Visser, T., Tak, T., Lammers, J.W. et al. (2012). A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *Journal of Clinical Investigation*, 122 (1), 327-336.

Pinheiro, D., Singh, Y., Grant, C.R., Appleton, R.C., Sacchini, F., Walker, K.R., et al. (2011). Phenotypic and functional characterization of a CD4(+)CD25(high) FOXP3(high) regulatory T-cell population in the dog. *Immunology*, 132 (1), 111-122.

Pinto, L.H., Canary, P.C., Araújo, C.M., Bacelar, S.C. and Souhami, L. (1991). Prospective randomized trial comparing hyperfractionated versus conventional radiotherapy in stages III and IV oropharyngeal carcinoma. *International Journal of Radiation Oncology Biology Physics,* 21 (3), 557-562.

Pitiyage, G., Tilakaratne, W.M., Tevassoli, M. and Warnakulasuriya, S. (2009). Molecular markers in oral epithelial dysplasia. Review. *Journal of Oral Pathology & Medicine*, 38 (10), 737-752.

Porakishvili, N., Mageed, R., Jamin, C., Pers, J.O., Kulikova, N., Renaudineau, Y., et al. (2001). Recent progress in the understanding of B-cell functions in autoimmunity. *Scandinavian Journal of Immunology,* 54 (1-2) 30-38.

Porrata, L.F., Ristow, K., Habermann, T., Inwards, D.J., Micallef, I.N. and Markovic, S.N. (2010). Predicitng survival for diffuse large B-cell lymphoma patients using baseline neutrophil/lymphocyte ratio. *American Journal of Haematology*, 85 (1), 896-899.

Porter, C.M., Horvath-Arcidiacono, J.A., Singh, A.K., Horvath, K.A., Blood, E.T. and Mohiuddin, M.M. (2007). Characterization and expansion of baboon CD4+CD25+ Treg cells for potential use in a nonhuman primate xenotransplantation model. *Xenotransplantation*, 14, 298-308.

Pot C., Apetoh L. and Kuchroo V.K. (2011). Type 1 regulatory T cells (Tr1) in autoimmunity. *Seminars in Immunology*, 23 (3), 202-208.

Poulsen, M.G., Denham, J.W., Peter,s L.J., Lamb, D.S., Spry, N.A., Hindley, A., et al. (2001). A randomised trial of accelerated and conventional radiotherapy for stage III and IV squamous carcinoma of the head and neck: A Trans-Tasman Radiation Oncology Group Study. *Radiotherapy & Oncology*, 60 (2), 113-122.

Proctor, M.J., Morrison, D.S., Talwar, D., Balmer, S.M., Fletcher, C.D., O'Reilly, D.S., et al. (2011). A comparison if inflammation based prognostic scores in patients with cancer. A Glasgow Inflammation Outcome Study. *European Journal of Cancer,* 47 (17), 2633-2641.

Proctor, M.J., Horgan, P.G., Tawlar, D., Flectcer, C.D., Morrison, D.S. and McMillan D.C. (2013). Optimization of the systemic inflammation-based Glasgow prognostic score: a Glasgow Inflammation Outcome Study. *Cancer,* 119 (12), 2325-2332.

Proulx, D.R., Ruslander, D.M., Dodge, R.K., Hauck, M.L., Williams, L.E., Hom, B., et al. (2003). A retrospective analysis of 140 dogs with oral melanoma treated with external beam irradiation. *Veterinary Radiology & Ultrasound*, 44 (3), 352-359.

Pugh, C.W. and Ratcliffe, P.J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature Medicine*, 9 (6), 677-684.

Quian, X. and Tuszynski, G.P. (1996). Expression of thrombospodonin 1 in cancer: a role in tumour progression. *Proceedings of the Society for Experimental Biology and Medicine*, 212 (3), 199-207.

R Core Team. (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Rai, B. and Kharb, S. (2008). Effect of Scaling and Root Planning in Periodontitis on Peripheral Blood. *International Journal of Developmental Science*, 6, 1.

Rak, J., Mitsuhashi, Y., Bayko, L., Filmus, J., Shirasawa, S., Sasazuki, T. and Kerbel, R.S. (1995). Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Research*, 55 (20), 4575-1580.

Ramsdell, F. and Ziegler, S.F. (2014). FOXP3 and scurfy. How it all began. *Nature Reviews Immunology,* 14 (5), 343-349.

Rassnick, K.M., Goldkamp, C.E., Erb, H.N., Scrivani, P.V., Njaa, B.L., Gieger, T.L., et al. (2006). Evaluation of factors associated with survival in dogs with untreated nasal carcinomas: 139 cases (1993-2003). *Journal of American Veterinary Medical Association*, 229 (3), 401-406.

Rastinejad, F., Polverini, P.J. and Bouck, N.P. (1989). Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell*, 56 (3), 345-355.

Raungkaewmanee, S., Tangjitgamol, S., Manusirivithaya, S., Srijaipracharoen, S. and Thavaramara, T. (2012). Platelet to lymphocyte ratio as a prognostic factor for epithelial ovarian cancer. *Journal of Gynecologic Oncology*, 23 (4), 265-273.

Rawlings, N.G., Simko, E., Bebchuk, T., Caldwell, S.J. and Singh, B. (2003). Localization of integrin αvβ3 and vascular endothelial growth factor receptor-2 (KDR/Flk-1) in cutaneous and oral melanomas of dog. *Histology and Histopathology*, 18 (3), 819-826.

Rawlinson, J.E., Goldstein, R.E., Reiter, A.M., Attwater, D.Z. and Harvey, CE. (2011). Association of periodontal disease with systemic health indices in dogs and the systemic response to treatment of periodontal disease. *Journal of American Veterinary Medical Association*, 238 (5), 601-609.

Ray-Coquard, I., Cropet, C., Van Glabbeke, M., Sebban, C., Le Cesne, A., Judson, I., et al. (2009). European Organization for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group. Lymphopaenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas and lymphomas. *Cancer Research*, 69 (13), 5383-5391.

Read, S., Malmstrom, V. and Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD4+CD25+ regulatory T cells that control intestinal inflammation. *Journal of Experimental Medicine*, 192 (2), 295-302.

Rebuzzi, L., Willmann, M., Sonneck, K., Gleixner, K.V., Florian, S., Rudin, K., Mayerhofer, M., et al. (2007). Detection of vascular endothelial growth factor (VEGF) and VEGF receptors Flt-1 and KDR in canine mastocytoma cells. *Veterinary Immunology and Immunopathology,* 115 (3-4), 320-333.

Rejec, A., Butinar, J., Pilla, T. and Benoit J. (2015). Diagnostic and prognostic significance of complete blood count indices (N/L, P/L, MPV/PLT, PLCRi) in canine cancer patients. In: Proceedings of the 2015 Annual Congress of the European Society of Veterinary Oncology, 28th to 30th of May 2015, Krakow, Poland. European Society of Veterinary Oncology, 2015, pp. 63.

Restucci, B., Maiolino, P., Paciello, O., Martano, M., De Vico, G. and Paparella, S. (2003). Evaluation of angiogenesis in canine seminomas by quantitative immunohistochemistry. *Journal of Comparative Pathology*, 128 (4), 252-259.

Restucci, B., Borzacchiello, G., Maiolino, P., Martano, M., Paciello, O. and Papparella, S. (2004). Expression of vascular endothelial growth factor receptor Flk-1 in canine mammary tumors. *Journal of Comparative Pathology*, 130 (2-3), 99-104.

Reuter, C.W., Morgan, M.A. and Eckardt, A. (2007). Targeting EGF receptor signalling in squamous cell carcinomas of the head and neck. *British Journal of Cancer*, 96 (3), 408-416.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature*, 386 (6626), 671-674.

Risau, W. and Flamme, I. (1995). Vasculogenesis. *Annual Review of Cell and Developmental Biology,* 11, 73-91.

Rissetto, K.C., Rindt, H., Selting, K.A., Villamil, J.A., Henry, C.J. and Reinero, C.R. (2010). Cloning and expression of canine CD25 for validation of an anti-human CD25 antibody to compare T regulatory lymphocytes in healthy dogs and dogs with osteosarcoma. *Veterinary Immunology Immunopathology,* 135, 137-145.

Robertson, S.J. and Hasenkrug, K.J. (2006). The role of virus-induced regulatory T cells in immunopathology. *Springer Seminars in Immunopathology*, 28 (1), 51-62.

Rogers, S.J., Harrington, K.J., Rhys-Evans, P., O-Charoenrat, P. and Eccles, S.A. (2005). Biological significance of c-erbB family oncogenes in head and neck cancer. *Cancer and Metastasis Reviews*, 24 (1), 47-69.

Roncarolo, M.G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K. and Levings, M.K. (2006). Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews*, 212 (1), 28-50.

Roxburgh, C.S. and McMillan, D.C. (2014). Cancer and systemic inflammation: treat the tumour and treat the host. *British Journal of Cancer*, 110 (6), 1409-1412.

Roxburgh, C.S. and McMillan, D.C. (2010). Role of systemic inflammatory response in predicting survival in patients with primary operable cancer. *Future Oncology*, 6 (1), 149-163.

Rydén, L., Linderholm, B., Nielsen, N.H., Emdin, S., Jönsson, P.E. and Landberg, G. (2003). Tumour specific VEGF-A and VEGFR-2/KDR protein are co-expressed in breast cancer. *Breast Cancer Research and Treatment*, 82 (3), 147-154.

Sagiv, J.Y., Michaeli, J., Assi, S., Mishalian, I., Kisos, H., Levy, L., et al. (2015). Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Reports*, 10 (4), 562-573.

Salehi, N., Eskandarian, R., Sanati, H.R., Firouzi, A., Shakerian, F., Abdi, S., et al. (2013). White blood cell count predicts increased mortality in acute myocardial infarction. *World Journal of Cardiovascular Diseases*, 3: 458-463.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alphachains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of Immunology*, 155 (3), 1151-1164.

Sakaguchi, S., Sakaguchi, N., Shimizu, J., Yamazaki, S., Sakihama, T., Itoh, M.T., et al. (2001). Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumour immunity, and transplantation tolerance. *Immunological Reviews*, 182, 18-32.

Sakaguchi, S., Setoguchi, R., Yagi, H. and Nomura, T. (2006). Naturally arising FoxP3- expressing CD25+CD4+ regulatory T cells in self-tolerance and autoimmune disease. *Current topics in microbiology and immunology*, 305, 51-66.

Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P. and Yamaguchi, T. (2009). Regulatory T cells: How do they suppress immune responses? *International Immunology*, 21 (10), 1105-1111.

Sakaguchi, S., Wing, K. and Yamaguchi, T. (2009). Dynamics of peripheral tolerance and immune regulation mediated by Treg. *European Journal of Immunology*, 39 (9), 2331-2336.

Sakka, V., Tsiodras, S., Giamarellos-Bourboulis, E.J. and Giamarellou, H. (2006). An upadate on the etiology and diagnostic evaluation of leukemoid reaction. *European Journal of Internal Medicine,* 17 (6), 394-398.

Salomon, B., Lenschow, D.J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A. and Bluestone J.A. (2000). B7/CD28 co-stimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity*, 12 (4), 431-440.

Samoszuk, M. (1997). Eosinophils and human cancer. *Histololgy and Histopathology*, 12 (3), 807-812.

Sasaki, H., Okamatsu, Y., Kawai, T., Kent, R., Taubman, M. and Stashenko, P. (2004). The interleukin 10 knockout mouse is highly susceptible to Porphyromonas gingivalis induced alveolar bone loss. *Journal of Periodontal Research*, 39 (6), 432-441.

Sato, H. and Takeda, Y. (2009). VEGFR-2 expression and relationship between tumour neovascularisation and histologic characteristics in oral squamous cell carcinoma. *Journal of Oral Science*, 51 (4), 551-557.

Saxlin, T., Ylöstalo, P., Suominen-Taipale, L., Männistö, S. and Knuuttila, M. (2011): Association between periodontal infection and obesity: Results of the health 2000 survey. *Journal of Clinical Periodontology*, 38 (3), 236-242.

Scaldaferri, F., Vetrano, S., Sans, M., Arena, V., Straface, G., Stigliano, G., et al. (2009). VEGF-A links angiogenesis and inflammation in inflammatory bowel disease pathogenesis. *Gastroenterology*, 136 (2), 585-595.

Scase, T.J., Edwards, D., Miller, J., Henley, W., Smith, K., Blunden, A. and Murphy, S. (2006). Canine mast cell tumors: correlation of apoptosis and proliferation markers with prognosis. *Journal of Veterinary Internal Medicine*, 20 (1), 151-158.

Schmidt, H., Bastholt, L., Geertsen, P., Christensen, I.J., Larsen, S., Gehl, J. and von der Maase, H. (2005). Elevated neutrophil and monocyte counts in peripheral blood are associated with poor survival in patients with metastatic melanoma: a prognostic model. *British Journal of Cancer,* 93 (3): 273-278.

Schmidt, H., Suciu, S., Punt, C.J., Gore, M., Kruit, W., Patel, P., et al. (2007). American Joint Committee on Cancer Stage IV Melanoma; EORTC 18951. Pretreatment levels of peripheral neutrophils and leucocytes as independent predictors of overall survival in patients with American Joint Committee on Cancer Stage IV Melanoma: Results of the EORTC 18951 biochemotherapy trial. *Journal of Clinical Oncology*, 25, 1562-1569.

Schniewind, B., Christgen, M., Hauschild, A., Kurdow, R., Kalthoff, H. and Klomp, HJ. (2005). Paraneoplastic leukemoid reaction and rapid progression in a patient with malignant melanoma: establishement of KT293, a novel G-CSF-secreting melanoma cell line. *Cancer Biology and Therapy,* 4 (1), 23-27.

Seibel, H., Stimmer, L., Siebert, U. and Beineke, A. (2010). Immunohistochemical characterization of selected cell markers for the detection of hematopoietic cells in formalin-fixed, paraffin wax-embedded lymphoid tissues of harbor seals (Phoca vitulina) and walruses (Odobenus rosmarus rosmarus). *Veterinary Immunology and Immunopathology*, 137 (3-4), 305-309.

Seiwert, T.Y., Salama, J.K. and Vokes, E.E. (2007). The chemoradiation paradigm in head and neck cancer. *Nature Clinical Practice Oncology*, 4 (3), 156-171.

Semenza, G.L. and Wang, G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Cellular and Molecular Biology*, 12, 5447-5454.

Semenza, GL. (2004). Intratumoural hypoxia, radiation resistance and HIF-1. Cancer Cell, 5 (5), 405-406.

Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S. and Dvorak, H.F. (1983). Tumour cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*, 219 (4587), 983-985.

Senger, D.R., Van de Water, L., Brown, L.F., Nagy, J.A., Yeo, K.T., Yeo, T.K., et al. (1993). Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer metastasis reviews*, 12 (3-4): 303-324.

Seo, K.S., Davis, W.C., Hamilton, M.J., Park, Y.H. and Bohach, G.A. Development of monoclonal antibodies to detect bovine FOXP3 in PBMCs exposed to a staphylococcal superantigen. *Veterinary Immunology and Immunopathology*, 2009; 128(1-3): 30-36.

Seretis, C., Seretis, F., Lagoudianakis, E., Politou, M., Gemenetzis, G. and Salemis, N.S. (2012). Enhancing the accuracy of platelet to lymphocyte ratio after adjustment

for large platelet count: a pilot study in breast cancer patients. *International Journal of Surgical Oncology*, 653608, 7 pages.

Seto, T., Higashiyama, M., Funai, H., Imamura, F., Uematsu, K., Seki, N., et al. (2006). Prognostic values of expression of vascular endothelial growth factor and its flt-1 and KDR receptors in stage 1 non-small-cell lung cancer. *Lung Cancer*, 53 (1), 91-96.

Seymour, G.J., Gemmell, E., Reinhardt, R.A., Eastcott, J. and Taubman, M.A. (1993). Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. *Journal of Periodontal Research*, 28 (6), 478-486.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L. and Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*, 376 (6535), 62-66.

Shao, B., Wahrenbrock, M.G., Yao, L., David, T., Coughlin, S.R., Xia, L., Varki, A. and McEver R.P. (2011). Carcinoma mucins trigger reciprocal activation of platelets and neutrophils in a murine model of Troussaeu syndrome. *Blood*, 118 (15), 4015-4023.

Sharaiha, R.Z., Halazun, K.J., Mirza, F., Port, J.L., Lee, P.C., Neugut, A.I., et al. (2012). Elevated preoperative neutrophil/lymphocyte ratio as a predictor of postoperative disease recurrence in esophageal cancer. *Annals of Surgical Oncology*, 18 (12): 3362-3369.

Sharma, D., Brummel-Ziedins, K.E., Bouchard, B.A. and Holmes, C.E. (2014). Platelets in tumour progression: a host factor that offers multiple potential targets in the treatment of cancer. *Journal of Cellular Physiology,* 229 (8), 1005-1015.

Shevach, E.M. (2002). CD4+CD25+ suppressor T cells: more questions than answers. *Nature Reviews Immunology*, 2 (6), 389-400.

Shevach, E.M. (2006). From vanilla to 28 flavours: multiple varieties of T regulatory cells. *Immunity,* 25 (2), 195-201.

Shibuya, K., Mathers, C.D., Boschi-Pinto, C., Lopez, A.D. and Murray, C.J. (2002). Global and regional estimates of cancer mortality and incidence by site: II. Results for the global burden of disease 2000. *BMC Cancer*, 2:37.

Shibuya, M. and Claesson-Welsh, L. (2006). Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Experimental Cell Research*, 312 (5), 549-560.

Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y. and Sakaguchi, S. (2002). Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. *Nature Immunology,* 3 (2), 135-142.

Shiomitsu, K., Johnson, C.L., Malarkey, D.E., Pruitt, A.F. and Thrall, D.E. (2009). Expression of epidermal growth factor receptor and vascular endothelial growth

factor in malignant canine epithelial nasal tumours. *Veterinary Comparative Oncology*, 7 (2): 106-114.

Shweiki, D., Itin, A., Soffer, D. and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*, 359 (6398), 843-845.

Silva, N., Abusleme, L., Bravo, D., Dutzan, N., Garcia-Sesnich, J., Vernal, R., Hernández, M. and Gamonal, J. (2015). Host response mechanisms in periodontal diseases. *Journal of Applied Oral Science*, 23 (3), 329-355.

Silva-Campa, E., Flores-Mendoza, L., Resendiz, M., Pinelli-Savedra, A., Mata-Haro, V., Mwangi, W. and Hernendez, J. (2009). Induction of T helper 3 regulatory cells by dendritic cells infected with porcine reproductive and respiratory syndrome virus. *Virology,* 387 (2): 373-379.

Simiantonaki, N., Taxeidis, M., Jayasinghe, C. and Kirkpatrick, C.J. (2007). Epithelial expression of VEGF receptors in colorectal carcinomas and their relationship to metastatic status. Anticancer Research, 27 (5A), 3245-3250.

Singh, A.J., Meyer, R.D., Band, H. and Rahimi, N. (2005). The caboxyl terminus of VEGFR-2 is required for PKC-mediated down-regualtion. Molecular Biology of the Cell, 16 (4), 2106-2118.

Singh, A.K., Horvath, K.A. and Mohiuddin, M.M. (2009). Rapamycin promotes the enrichment of CD4(+)CD25(hi)FoxP3(+) T regulatory cells from naive CD4(+) T cells of baboon that suppress antiporcine xenogenic response in vitro. *Transplantation Proceedings*, 41 (1), 418-421.

Sittel, C., Ruiz, S., Volling, P., Kvasnicka, H.M., Jungehülsing, M. and Eckel, H.E. (1999). Prognostic significance of Ki-67 (MIB1), PCNA and p53 in cancer of the oropharynx and oral cavity. *Oral Oncology*, 35 (6), 583-589.

Skaleric, U., Kramar, B., Petelin, M., Pavlica, Z. and Wahl, S.M. (1997). Changes in TGF-β1 levels in gingiva and serum associated with periodontal inflammation in humans and dogs. *European Journal of Oral Sciences*, 105 (2), 136-142.

Skladowski, K., Maciejewski, B., Golen, M., Pilecki, B., Przeorek, W. and Tamawski, R. (2000). Randomized clinical trial on 7-day-continuous accelerated irradiation (CAIR) of head and neck cancer-report on 3-year tumour control and normal tissue toxicity. *Radiotherapy & Oncology*, 55 (2), 101-110.

Slauther, D.L., Southwick, H.W. and Smejkal, W. (1953). Field cancerization in oral stratified squamous epithelium: clinical implication of multicentric origin. *Cancer*, 6, 963-968.

Sledzinska, A., Hemmers, S., Mair, F., Gorka, O., Ruland, J., Fairbairn, L., et al. (2013). TGF-ß is required for CD4+ T cell homeostasis but dispensable for regulatory T cell function. *PLoS Biol,* 11 (10): e1001674.

Smith, E.L., Finney, H.M., Nesbitt, A.M., Ramsdell, F. and Robinson, M.K. (2006). Splice variants of human FOXP3 are functional inhibitors of human CD4+ T cell activation. *Immunology*, 119 (2), 203-211.

Smith, R.A., Ghaneh, P., Sutton, R., Raraty, M., Campbell, F. and Neoptolemos, J.P. (2008). Prognosis of resected ampullary adenocarcinoma by preoperative serum CA19-9 levels and platelet- lymphocyte ratio. *Journal of Gastrointestinal Surgery*, 12 (8), 1422-1428.

Smith, R.A., Bosonnet, L., Raraty, M., Sutton, R., Neoptolemos, J.P., Campbell, F. and Ghaneh, P. (2009). Preoperative platelet-lymphocyte ratio is an independent significant prognostic marker in resected pancreatic ductal adenocarcinoma. *American Journal of Surgery*, 197 (4), 466-472.

Song, X., Li, B., Xiao, Y., Chen, C., Wang, Q., Liu, Y., et al. (2012). Structural and biological features of FOXP3 dimerization relevant to regulatory T cell function. Cell Reports, 28, 1(6), 665-675.

Srinivasan, P.C. (2013). Neutrophils – the sentinels of periodontal innate immunity. *Journal of clinical and cellular immunology*, S13:002.

Stetler-Stevenson, W. (2008). The tumor microenvironment: regulation by MMP-independent effects of tissue inhibitor of matrix metalloproteinase-2. *Cancer Metastasis Reviews,* 27 (1), 57-66.

Stinga, A.C., Margaretiescu, O., Stinga, A.S., Pirici, D., Ciurea, R., Bunget, A. and Cruce, M. (2011). VEGFR-1 and VEGFR-2 immunohistochemical expression in oral squamous cell carcinoma: a morphometric study. *Romanian Journal of Morphology and Embryology*, 52 (4): 1269-1275.

Stoufi, E.D., Taubman, M.A., Ebersole, J.L., Smith, D.J. and Stashenko, P.P. (1987). Phenotypic analyses of mononuclear cells recovered from healthy and diseased human periodontal tissues. *Journal of Clinical Immunology,* 7 (3), 235-245.

Strober, S., Cheng, L., Zeng, D., Palathumpat, R., Dejbakhsh-Jones, S., Huie, P. and Sibley, R. (1996). Double negative (CD4-CD8-αβ+) T cells which promote tolerance induction and regulate autoimmunity. *Immunological Reviews*, 149, 217-230.

Summers, C., Rankin, S.M., Condliffe, A.M., Singh, N., Peters, A.M. and Chilvers, E.R. (2010). Neutrophil kinetics in health and disease. *Trends in Immunology*, 31 (8), 318-324.

Symbas N.P., Townsend M.F., El-Galley R., Keane T.E., Graham S.D. and Petros J.A. (2000). Poor prognosis associated with thrombocytosis in patients with renal cell carcinoma. *BJU International*, 86 (3), 203-207.

Syrjänen, S., Markkanen, H. and Syrjänen, K. (1984). Inflammatory cells and their subsets in lesions of juvenile periodontitis. A family study. *Acta Odontologica Scandinavica*, 42 (5), 285-292.

Swartz, J.E., Pothen, A.J., Stegman, I., Willems, S.M. and Grolman, W. (2015). Clinical implications of hypoxia biomarker expression in head and neck squamous cell carcinoma: a systematic review. *Cancer Medicine,* 4 (7), 1101-1116.

Tabeta, K., Yamazaki, K., Hotokezaka, H., Yoshie, H. and Hara, K. (2000). Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. *Clinical And Experimental Immunology*, 120 (2), 285-293.

Takahashi, K., Nagai, A., Satoh, N., Kurihara, H. and Murayama, Y. (1995). Studies on the phenotypic and functional characterization of peripheral blood lymphocytes from patients with early-onset periodontitis. *Journal of Periodontology*, 66, 391-396.

Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, M., Itoh, M., Iwata, M., et al. (1998). Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *International Immunology*, 10, 1969-1980.

Takahashi, T., Yamaguchi, S., Chida, K. and Shibuya, M. (2001). A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. *European Molecular Biology Organisation Journal*, 20 (11), 2768-2778.

Takahashi, Y., Kitadai, Y., Bucana, C.D., Cleary, K.R. and Ellis, L.M. (1995). Expression of vascular endothelial growth factor and its receptors, KDR, correlates with vascularity, metastases and proliferation of human colon cancer. *Cancer Research,* 55 (18), 3964-3968.

Takeichi, O., Haber, J., Kawai, T., Smith, D.J., Moro, I. and Taubman, M.A. (2000). Cytokine profiles of T-lymphocytes from gingival tissues with pathological pocketing. *Journal of Dental Research*, 79 (8), 1548-1555.

Takeuchi, Y., Yoshie, H. and Hara, K. (1991). Expression of interleukin-2 receptor and HLA-DR on lymphocyte subsets of gingival crevicular fluid in patients with periodontitis. *Journal of Periodontal Research*, 26 (6), 502-510.

Tang, Q., Henriksen, K.J., Boden, E.K., Tooley, A.J., Ye, J., Subudhi, S.K., Zheng, X.X., et al. (2003). CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *Journal of Immunology*, 171 (7), 3348-3352.

Tanno, S., Ohsaki, Y., Nakanishi, K., Toyoshima, E. and Kikuchi, K. (2004). Human cell lung cancer cells express functional VEGF receptors, VEGFR-2 and VEGFR-2. Lung Cancer, 46 (1), 11-19.

Tang, Q., Boden, E.K., Henriksen, K.J., Bour-Jordan, H., Bi M. and Bluestone, JA. (2004). Distinct roles of CTLA-4 and TGF-β in CD4+CD25+ regulatory T cell function. *European Journal of Immunology*, 34 (11), 2996- 3005.

Taubman, M.A., Stoufi, E.D., Ebersole, J.L. and Smith, D.J. (1984). Phenotype studies of cells from periodontal tissues. *Journal of Periodontal Research*, 19 (6), 587-590.

Taylor, G.W. and Borgnakke, W.S. (2008). Periodontal disease: Associations with diabetes, glycemic control and complications. *Oral Diseases*, 14 (3), 191-203.

Teles, R., Teles, F., Frias-Lopez, J., Paster, B. and Haffajee, A. (2013). Lessons learned and unlearned in periodontal microbiology. *Periodontology 2000*, 62 (1), 95-162.

Teramukai, S., Kitano, T., Kishida, Y., Kawahara, M., Kubota, K., Komuta, K., et al. (2009). Pretreatment neutrophil count as an independent prognostic factor in advanced non-small-cell lung cancer: an analysis of Japan Multinational Trial Organization LC00-03. European Journal of Cancer, 45 (11), 1950-1958.

Tefferi, A. (2005). Blood eosinophilia: new paradigm in disease classification, diagnosis and treatment. *Mayo Clinic Proceedings*, 80 (1), 75-83.

Teng, Y.T., Taylor, G.W., Scannapieco, F., Kinane, D.F., Curtis, M., Beck, J.D. and Kogon, S. (2002). Periodontal health and systemic disorders. *Journal of the Canadian Dental Association*, 68 (3), 188-192.

Tenorio, A.R., Spritzler, J., Martinson, J., Gichinga, C.N., Pollard, R.B., Lederman, M.M. et al. (2009). The effect of aging on T-regulatory cell frequency in HIV infection. Clinical Immunology, 130 (3), 298-303.

Thamm, D.H., Rose, B., Kow, K., Humbert, M., Mansfield, C.D., Moussy, A., et al. (2012). Masitinib as a chemosensitizer of canine tumour cells: a proof of concept study. *Veterinary Journal,* 191 (1), 131-134.

Theon, A.P., Madewell, B.R., Harb, M.F. and Dungworth, D.L. (1993). Megavoltage irradiation of neoplasms of the nasal and paranasal cavities in 77 dogs. *Journal of American Veterinary Medical Association*, 202 (9), 1469-1475.

Theon, A.P., Rodriguez, C. and Madwell, B.R. (1997). Analysis of prognostic factors in patterns of faliure in dogs with malignant oral tumours treated with megavoltage irradiation. *Journal of American Veterinary Medical Association*, 210 (6), 785-788.

Thomas, B., Ramesh, A. and Ritesh, K. (2006). Relationship between periodontitis and erythrocyte count. *Journal of Indian Society of Periodontology*, 10, 288-291.

Thompson, C.B., Jakubowski, J.A., Quinn, P.G., Dykin, D. and Valeri, C.R. (1984). Platelet size and age determine platelet function independently. *Blood*, 64 (6), 1372-1375.

Thornton, A.M. and Schevach, E.M. (1998). CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin-2 production. *Journal of Experimental Medicine*, 188 (2), 287-296.

Thrall, D.E., McEntee, M.C. and Novotney, C. (1993). A boast technique for irradiation of malignant canine nasal tumours. *Veterinary Radiology & Ultrasound,* 34, 295-300.

Tizard, I.R. (2012). Regulation of adaptive immunity. In: Veterinary Immunology; 9th ed., Elsevier,pp 209-224.

Toi, M., Kashitani, J. and Tominaga, T. (1993). Tumor angiogenesis is an independent prognostic indicator in primary breast carcinoma. *International Journal of Cancer*, 55 (3), 371-374.

Tominaga, M., Horiuchi, Y., Ichikawa, M., Yamashita, M., Okano, K., Jikumaru, Y., et al. (2010). Flow cytometric analysis of peripheral blood and tumor-infiltrating regulatory T cells in dogs with oral malignant melanoma. *Journal of Veterinary Diagnostic Investigation*, 22 (3), 438-441.

Trott, K. and Kummermehr J. (1985). What is known about tumour proliferation rates to choose between accelerated fractionation or hyperfractionation? *Radiotherapy & Oncology*, 3 (1), 1-9.

Tumuluri, V., Thomas, G.A. and Fraser, I.S. (2002). Analysis of the Ki-67 antigen at the invasive tumour front of human oral squamous cell carcinoma. *Journal of Oral Pathology & Medicine*, 31 (10), 598-604.

Turek, M.M. and Lana, S.E. (2007). Canine sinonasal tumours. In: Withrow SJ, Vail DM, eds. Small Animal Clinical Oncology, 4th Ed. Philadelphia: WB Saunders, pp 525-539.

Urie, B.K., Russe, D.S., Kissberth, W.C. and London, C.A. (2012). Evaluation of the expression and function of vascular endothelial growth factor receptor 2, platelet derived growth factor receptors – alpha and beta, KIT and RET in canine apocrine gland anal sac adenocarcinoma and thyroid carcinoma. *BMC Veterinary Research*, 8, 67.

Van Diest, P.J., Brugal, G. and Baak, J.P. (1998). Proliferation markers in tumours: interpretation and clinical value. *Journal of Clinical Pathology*, 51 (10), 716-724.

Vahlenkamp, T.W., Tompkins, M.B. and Tompkins, W.A. (2004). Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. *Journal of Immunology*, 172 (8), 4752-4761.

Valencia, X., Yarboro, C., Illei, G. and Lipsky, P.E. (2007). Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. *Journal of Immunology*, 178 (4), 2579-2588.

Veenhof, E.Z., Rutten, V.P., Van Noort, R., Knol, E.F. and Willemse, T. (2010). Evaluation of T-cell activation in the duodenum of dogs with cutaneous food hypersensitivity. *American Journal of Veterinary Research*, 71 (4), 441-446.

Veenhof, E.Z., Knol, E.F, Schlotter, Y.M., Vernooij, J.C., Rutten, V.P. and Willemse T. (2011). Characterisation of T cell phenotypes, cytokines and transcription factors in the skin of dogs with cutaneous adverse food reactions. *Veterinary Journal*, 87 (3), 320-324.

Venables, W.N. and Ripley, B.D. (2002) Modern Applied Statistics with S. Fourth Edition. Springer, New York.

Veterinary cooperative oncology group - common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats. *Veterinary and Comparative Oncology* 2011. doi: 10.1111/j.1476-5829.2011.00283.

Vial, J., Isaacs, J.D. and Anderson, A.E. (2009). Regulatory T cells and autoimmunity. *Current Opinion in Hematology***,** 16 (4), 274-279.

Vokes, E.E., Weichselbaum, R.R., Lippman, S.M. and Hong, W.K. (1993). Head and neck cancer. *The New England Journal of Medicine*, 328 (3), 184-194.

Vignali, D.A., Collinson, L.W. and Workman, C.J. (2008). How regulatory T cells work. *Nature Reviews Immunology*, 8 (7): 523-532.

Wakai, K., Kawamura, T., Umemura, O., Hara, Y., Machida, J., Anno, T., et al. (1999). Associations of medical status and physical fitness with periodontal disease. *Journal of Clinical Periodontology*, 26 (10), 664-672.

Waldner, M.J., Wirtz, S., Jefremow, A., Warntjen, M., Neufert, C., Atreya, R., et al. (2010). VEGF receptor signaling link inflammation and tumorigenesis in colitis-associated cancer. J Exp Med, 207 (13), 2855-2868.

Walker, M.R., Kasprowicz, D.J., Gersuk, V.H., Benard, A., Van Landeghen, M, Buckner, J.H and Ziegler, S.F. (2003). Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *The Journal of Clinical Investigation,* 112 (9), 1437-1443.

Walsh, S.R., Cook, E.J., Goulder, F., Justin, T.A. and Keeling, N.J. (2005). Neutrophil- lymphocyte ratio as a prognostic factor in colorectal cancer. *Journal of Surgical Oncology*, 91 (3), 181-184.

Walter, J.W., North, P.E., Waner, M., Mizeracki, A., Blei, F., Walker, J.W., et al. (2002). Somatic mutation of vascular endothelial growth factor receptors in juvenile hemangioma. *Genes Chromosomes Cancer*, 33 (3), 295-303.

Wang, C.C. (1988). Local control of oropharyngeal carcinoma after two accelerated hyperfractionation radiation therapy schemes. *International Journal of Radiation Oncology, Biology, Physics,* 14 (6), 1143-1146.

Wang, G.L. and Semenza, G.L. (1993). General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proceedings of the National Academy of Sciences of the United States of America*, 90 (9), 4304-4308.

Watabe, A., Fukumoto, S., Komatsu, T., Endo, Y. and Kodosawa, T. (2011). Alterations in lymphocyte subpopulations in healthy dogs with aging and in dogs with cancer. *Veterinary Immunology and Immunopathology*, 142 (3-4), 189-200.

Webster, J.D., Yuzbasiyan-Gurkan, V., Miller, R.A., Kaneene, J.B. and Kiupel, M. (2007). Cellular Proliferation in Canine Cutaneous Mast Cell Tumors: Associations with c-KIT and Its Role in Prognostication. *Veterinary Pathology,* 44 (3), 298-308.

Weiden, P.L., Storb, R., Tsoi, M.S., Graham, T.C., Lerner, K.G. and Thomas, E.D. Infusion of donor lymphocytes into stable canine radiation chimeras: implications for mechanism of transplantation tolerance. *Journal of Immunology,* 1976; 116 (5), 1212-1219.

Weidner, N., Carroll, P.R., Flax, J., Blumenfeld, W. and Folkman, J. (1993). Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *The American Journal of Pathology*, 143 (2), 401-409.

Wein, R.O. and Weber, R.S. (2005). Malignant neoplasms of the oral cavity. In: Cummings C.W., Flint P.W., Harker L.A., Haughey B.H., Richardson M.A., Schuller D.E., eds. Cummings Otolaryngology: Head and Neck Surgery. 4th ed. St. Louis, Mo: Mosby Year Book; pp 1578-1617.

Wilson, D.W. and Dungworth, D.L. (2002). Tumours of the respiratory tract. In: Meuten, D.J., ed. Tumorus in domestic animals. 4th Ed. Oxford: Blackewell Publishing, pp 365-373.

Wing K. and Sakaguchi S. (2010). Regulatory T cells exert checks and balances on self-tolerance and autoimmunity. *Nature Immunology*, 11 (1), 7-13.

Withers, H.R. (1985). Biologic basis for altered fractionation schemes. *Cancer*, 55 (9), 2086-2095.

Withrow, S.J. (2007). Tumours of the respiratory system. In: Withrow & MacEwen's Small Animal Clinical Oncology, 4th Edit., Withrow SJ, Vail DM, Eds. Saunders Elsevier, St Louis, pp 511-539.

Withrow, S.J. (2001). Cancer of gastrointestinal tract. In: Small Animal Clinical Oncology, SJ Withrow and EG MacEwen, eds. Philadelphia, Saunders, pp 305-317.

Wolfesberger, G., Tinar, Z., Witter, K., Guija de Arespachoaga, A., Skalicky, M., Walter, I., et al. (2008). Microvessel density in normal lymph nodes and lymphomas of dogs and their correlation with vascular endothelial growth factor expression. *Research in Veterinary Science*, 85: 56-61.

Wong, P. and Pamer, E.G. (2003). CD8 T cell responses to infectious pathogens. *Annual Review of Immunology*, 21: 29-70.

Wongyanin, P., Buranapraditkum, S., Chokeshai-Usaha, K., Tanawongiwech, R. and Suradhat, S. (2010). Induction of inducible CD4+CD25+Foxp3+ regulatory T

lymphocytes by porcine reproductive and respiratory syndrome virus (PRRSV). *Veterinary Immunology and Immunopathology*, 133 (2-4), 170-182.

Workman, C.J., Szymczak-Workman, A.L., Collison, L.W., Pillai, M.R. and Vignalli, D.A. (2009). The development and function of regulatory T cells. *Cell Mol Life Sci*, 66 (16), 2603-2622.

Yamamoto, S., Konishi, I., Mandai, M., Kuroda, H., Komatsu, T., Nanbu, K., et al. (1997). Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels. *British Journal of Cancer*, 76 (9), 1221-1227.

Yamamoto, M., Fujihashi, K., Hiroi, T., McGhee, J.R., Van Dyke, T.E., Kiyono, H. (1997). Molecular and cellular mechanisms for periodontal diseases: Role of Th1 and Th2 type cytokines in induction of mucosal inflammation. *Journal of Periodontal Research,* 32 (1 Pt 2), 115-119.

Yamazaki, K., Nakajima, T. and Hara, K. (1995). Immunohistological analysis of T cell functional subsets in chronic inflammatory periodontal disease. *Clinical and Experimental Immunology*, 99, 384-391.

Yamazaki, K., Ohsawa, Y., Tabeta, K., Ito, H., Ueki, K., Oda, T., Yoshie, H. and Seymour, G.J. (2002). Accumulation of human heat shock protein 60-reactive T cells in the gingival tissues of periodontitis patients. *Infection and Immunity*, 70 (5), 2492-2501.

Yilmaz, O. (2008). The chronicles of Porphyromonas gingivalis: the microbium, the human oral epithelium and theirinterplay. *Microbiology*, 154: 2897-2903.

Ylöstalo, P., Suominen-Taipale, L., Reunanen, A. and Knuuttila, M. (2008). Association between body weight and periodontal infection. *Journal of Clinical Periodontology*, 35 (4), 297-304.

Yonemaru, K., Sakai, H., Murakami, M., Yanai, T. and Masegi, T. (2006). Expression of vascular endothelial growth factor, basic fibroblast growth factor and their receptors (Flt-1, Flk-1, and Flg-1) in canine vascular tumors. *Veterinary Pathology*, 43 (6): 971-980.

Yu, J.L., Rak, J.W., Coomber, B.L., Hicklin, D.J. and Kerbel, RS. (2002). Effect of p53 status on tumour response to antiangiogenic therapy. *Science,* 295 (5559): 1526-1528.

Zhang, Z.F., Morgenstern, H., Spitz, M.R., Tashkin, D.P., Yu, G.P., Hsu, T.C. and Schantz, S.P. (2000). Environmental tobacco smoking, mutagen sensitivity, and head and neck squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev*, 9 (10), 1043-1049.

Zhang, S.C., Miyamoto, S., Kamijo, T., Hayashi, R., Hasebe, T., Ishii, G., et al. (2003). Intratumour microvessel density in biopsy specimens predicts local response

of hypopharyngeal cancer to radiotherapy. *Japanese Journal of Clinical Oncology*. 33 (12), 613-619.

Ziegler, S.F. (2006). FOXP3 of mice and men. *Annual Review of Immunology*, 24: 209-226.

Ziche, M. and Gullino, P.M. (1982). Angiogenesis and neoplastic progression in vitro. *Journal of the National Cancer Institute,* 69, 483-487.