Association Between Neutrophil Count and Inflammatory Markers in Canine Appendicular Osteosarcoma

A. Chau[1], V. Huntley[1], N. Hillis[1], R. Speare[2], C.R. Schott[2], R.D. Wood[2], A.J. Mutsaers[1], G.A. Wood[2], A. M. Viloria-Petit[1]

1. Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Canada
2. Department of Pathobiology, Ontario Veterinary College, University of Guelph, Canada

Background

Canine Osteosarcoma and Neutrophils

- Osteosarcoma (OSA) accounts for 80-90% of all malignant bone tumours in canines, with higher prevalence in middle-aged large and giant breeds, where it mainly affects the appendicular skeleton[1].
- OSA treatment includes a combination of surgery and adjuvant therapy (chemotherapy or radiation), but even with this, the average survival time is ~1 year[1].
- Inflammation is a hallmark of cancer, and neutrophils are key mediators of both acute and chronic inflammation[2].
- Neutrophils are well documented to mediate cancer progression via production of reactive oxygen species (ROS) and neutrophil extracellular traps (NETs). ROS can induce DNA and tissue damage, while NETs can serve as scaffolds for circulating tumour cells and provide a favorable environment for metastasis[2].
- Tumour and stroma cells release inflammatory markers to recruit immune cells (including neutrophils) to the site of inflammation[2] (**Table 1**). However, there is limited research on the recruitment of neutrophils to the tumour site in OSA.

Table 1. Inflammatory Markers and Neutrophil Recruitment

Inflammatory marker	Inflammation Type	Relationship to Neutrophils
CRP	Acute Inflammation	Recruits neutrophils through IL-8 production and complement system[3].
IL-8	Acute Inflammation	Neutrophils degranulation and recruitment[3].
IL-6	Acute and Chronic Inflammation	Promotes neutrophil egression from bone marrow[4]. Induce transcription and production of CRP[3].
IL-18	Acute and Chronic Inflammation	Promotes neutrophil activation via IL-18R[5]. Induces synthesis of TNF-α and GM-CSF[5].
GM-CSF	Acute and Chronic Inflammation	Promotes neutrophils differentiation, survival, and migration[6]. Primes other cells to enhance IL-8 production[6].
KC-Like	Acute and Chronic Inflammation	Neutrophils recruitment to site of inflammation via CXCR2 receptor and GAGs[7].
MCP-1	Acute and Chronic Inflammation	Neutrophils recruitment through chemotaxis. Recruits KC-like, MIP-2, and TNF-α[8].
IP-10	Acute Inflammation	Neutrophils are a significant source of IP-10 and acts via CXCR3 receptor[9].

CRP = C-reactive protein, IL-8 = Intereukin-8, GM-CSF = Granulocyte-macrophage colony-stimulating factor, KC-like = Keratinocyte chemotactic-like, MCP-1 = Monocyte chemoattractant protein-1, IP-10 = Interferon-γ-induced protein 10

Prognostic Value of Circulating Segmented Neutrophils and Cytokines

We previously observed that a high count of circulating segmented neutrophils (SN) correlated with reduced time to metastasis and overall survival in canine OSA patients. Independently, we found similar associations for IP-10 and IL-8. Several other circulating inflammatory markers were dysregulated in OSA patients compared to healthy controls.

Objectives

Objective 1: Circulating Segmented Neutrophils (SN) and Cytokines

Test the correlation between 7 previously assessed inflammatory cytokines and SN count in a small cohort of canine OSA patients.

Objective 2: Circulating SN and C-reactive Protein (CRP)

Quantify CRP concentration and test correlation with SN count in canine OSA patients.

Methods

Patient population

Dogs presenting to the Ontario Veterinary College Health Sciences Centre (OVC-HSC) with diagnosis of appendicular OSA and receiving standard of care treatment. Patients were excluded for evident metastasis at diagnosis, diseases that impact leukocyte count, systemic inflammatory disease, GI issues, or history of corticosteroid treatment.

Objective 1: Circulating Segmented Neutrophils (SN) and Cytokines

Cytokines: IL-8, IL-6, IL-18, GM-CSF, KC-like, MCP-1, IP-10

Patient group: n=37 canine OSA patients with predetermined values of both cytokine levels and SN count from blood samples taken before surgery

Objective 2: Circulating SN and C-reactive Protein (CRP)

Patient group: n=13 patients to determine CRP concentration (7 with high and 6 with low SN count)

Fig. 1 ELISA Protocol

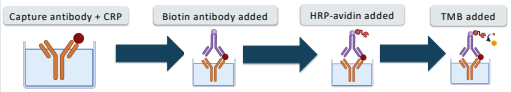


Fig. 1 Sandwich ELISA method used to determine CRP concentrations in serum samples (CUSABIO Biotech Co., Ltd., Texas, USA).

Statistical Analysis

Prism Software was used to test an association between SN count and all inflammatory markers (i.e. cytokines and CRP) using two methods. (1) Spearman correlation analysis was used as the data did not pass the Shapiro-Wilks Test for normality, and (2) Fisher's Exact Test was performed using optimal survival cut-points determined previously using X-Tile (Yale University, Connecticut, USA). $p<0.05$ was considered significant.

Results

Objective 1: Circulating Segmented Neutrophils (SN) and Cytokines

- Minimal correlation observed between SN count and cytokine level (**Fig. 2**)
- Negative correlation trend between SN count and IL-6, IL-18, KC-like, MCP-1, and IP-10

Correlation Graphs

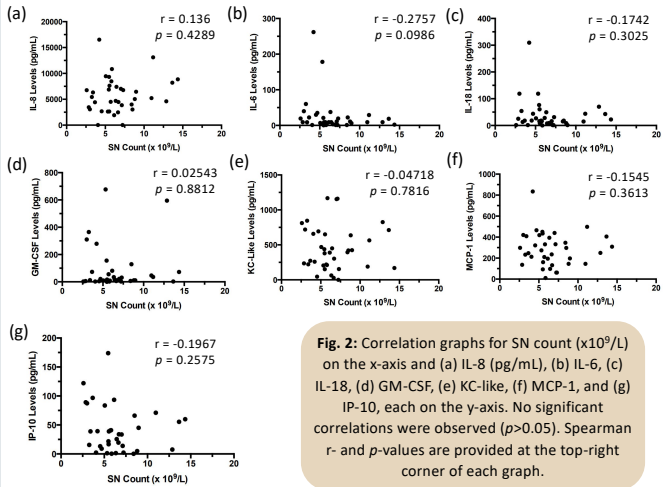


Fig. 2: Correlation graphs for SN count (x10⁹/L) on the x-axis and (a) IL-8 (pg/mL), (b) IL-6, (c) IL-18, (d) GM-CSF, (e) KC-like, (f) MCP-1, and (g) IP-10, each on the y-axis. No significant correlations were observed ($p>0.05$). Spearman r- and p-values are provided at the top-right corner of each graph.

Results

Fisher's Exact Test

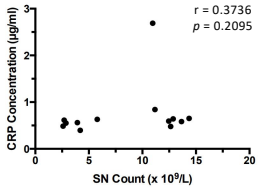
- Minimal association observed between SN count and all cytokines (**Table 2**).

Cytokine		IL-8		IL-6		IL-18		GM-CSF		KC-Like		MCP-1		IP-10	
		High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low
Neutrophil	High	4	5	0	9	0	9	1	8	9	0	1	8	5	3
	Low	12	15	3	25	1	27	5	23	22	6	7	21	7	20
P-value		1.000		0.5622		1.000		1.000		0.3025		0.6487		0.0912	

Table 1: Fisher's Exact Test between SN Count and each cytokine. Contingency table with number of patients in each group based on the optimal survival cut-point.

Objective 2: Circulating SN and C-reactive Protein (CRP)

Correlation Graph



- Minimal correlation observed between SN count and CRP concentrations (**Fig. 3**)
- All values fall within the range of normal CRP levels

Fig. 3: Correlation graph for SN count (x10⁹/L) on the x-axis and CRP concentration (μg/mL) on the y-axis. Correlation observed was not significant ($p>0.05$). Spearman r- and p-values are provided at the top-right corner of the graph.

Limitations

- Small sample size, which decreases the statistical power.
- CRP analysis was a preliminary study and will need to be repeated with the total number of patients.
- Serum cytokine levels do not necessarily represent the levels within the tumour microenvironment.

Conclusions & Future Work

- There were minimal association observed between SN count and all inflammatory markers in canine OSA using both a Spearman correlation and Fisher's Exact Test.
- P values for IL-6 with the Spearman test ($p = 0.0986$) and for IP-10 with the Fisher's Exact Test ($p = 0.0912$) suggests further investigation may be useful.
- Negative correlation trends observed with SN count and MCP-1, KC-Like, IL-6, and IP-10 levels potentially suggesting SNs are leaving circulation and entering tissue.
- In the future, we plan to investigate other systemic inflammatory signals (i.e. serum amyloid A) as it has shown prognostic value in OSA
- Investigating the levels of all cytokines in the tumour microenvironment will also inform their potential role in tumour progression.

Funding

- OVC Pet Trust
- Natural Sciences and Engineering Research Council of Canada (NSERC)

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