Evaluation of prognostic impact of pretreatment neutrophil to lymphocyte and lymphocyte to monocyte ratios in dogs with oral malignant melanoma treated with surgery and adjuvant CSPG4antigen electrovaccination: an explorative study

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(Article begins on next page)
Evaluation of prognostic impact of pre-treatment neutrophil to lymphocyte and lymphocyte to monocyte ratios in dogs with oral malignant melanoma treated with surgery and adjuvant CSPG4-antigen electrovaccination: an explorative study.

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The role of systemic inflammation in cancer’s progression has been widely investigated, especially in melanoma in humans. Pre-treatment leukocytes count and ratios play a recognized prognostic role in several types of malignancies, but no information is available regarding canine oral malignant melanoma (COMM). The purpose of this explorative retrospective study was to investigate the prognostic impact of pre-treatment neutrophil to lymphocyte (NLR) and lymphocyte to monocyte (LMR) ratios in dogs with oral malignant melanoma that underwent surgical resection and immunotherapy with adjuvant CSPG4-antigen electrovaccination. Thirty-nine dogs with histologically confirmed oral melanoma and with available pre-treatment haematological analyses, performed at maximum 60 days before the first treatment, were retrospectively enrolled. Statistical analysis was performed to explore possible correlations among NLR and LMR with age, clinical stage, tumour pigmentation, tumour size, nuclear atypia, mitotic index, Ki67, CSPG4 status, ulceration, bone invasion and excision margins status. The impact of NLR and LMR on overall survival time (OST) was explored among various ratio cut off and across different time points with Kaplan Meier method. No significant relationship was identified between leukocytes ratios and histological parameters, CSPG4, margins, age, tumour size and clinical stage. NLR and LMR did not display a prognostic impact on the survival time of the entire population. Pre-treatment leukocytes ratios may not represent a useful prognostic factor in dogs with oral melanoma, especially in absence of distant metastatic disease.

**KEYWORDS**

Canine oral melanoma, leukocytes ratio, prognosis, survival
**Introduction**

Malignant melanoma (MM) represents an aggressive disease in both human and canine patients. The oral cavity is the most common localization in dogs.\(^1,2\) Canine oral malignant melanoma (COMM) invades locally and is characterized by a high and early metastatic potential to regional lymph nodes and lungs, although other sites may also be affected. The clinical-biological behaviour of MM in dogs may be predicted based on prognostic factors including anatomic site, size and clinical stage.\(^3-5\) Other prognosticators include Ki67, mitotic index, nuclear atypia, degree of pigmentation and platelet derived growth factor receptor (PDGFR)-\(a/\beta\) co-expression.\(^6-8\) Conventional therapies such as surgery and/or radiation are finalised to local tumour control. Nevertheless, metastatic disease still remains the major cause of death\(^9,10\) with adjuvant dose-intense chemotherapy appearing to be not determinant in prolonging survival.\(^11-14\) For these reasons, and also considering the high immunogenicity of malignant melanoma,\(^15\) immunotherapy with different types of vaccine has been proposed as an adjuvant, systemic therapeutic tool, with encouraging results over the last years.\(^16-18\)

It is recognised that inflammation plays a central role in cancer development and progression.\(^19\) Systemic inflammation is associated with alterations in peripheral blood leukocytes that can be captured by the neutrophil to lymphocyte ratio (NLR).\(^20\) The prognostic impact of NLR has been reported in various human cancers including melanoma, for which an increased NLR has been associated with a shorter survival.\(^21,22\)

Specifically, lymphocytes are crucial for building an effective humoral and cellular response directed against the tumour; on the other way monocytes recruited within the tumour produce growth factors and cytokines leading to immunosuppression and angiogenesis. All this creates an optimal tumour microenvironment. Additionally, according to recent human
meta-analysis, lymphocyte to monocyte ratio (LMR) seems to be an independent predictor of survival in several solid (nasopharyngeal, gastrointestinal, urinary and lung cancers) and haematological (lymphoma) malignancies, with low LMR representing a negative prognostic factor.\textsuperscript{23,24} In veterinary medicine leukocytes counts and ratios have also been investigated as a potential prognostic and diagnostic biomarker for several malignancies including lymphoma, soft tissue sarcoma, osteosarcoma and mast cell tumours,\textsuperscript{25-30} but no information is available regarding COMM. In this explorative retrospective study, we aim to evaluate the potential prognostic impact of pre-treatment NLR and LMR in COMM, assuming that a greater value of NLR and lower value of LMR would be associated with a decreased survival. We also aim to establish an NLR and LMR cut-off associated with the outcome of malignant melanoma canine patients.

Materials and methods

Case selection

Medical records of client-owned dogs referred to the XXXXXXXX from September 2009 to December 2019 were retrospectively reviewed searching for animals affected by COMM. All the patients enrolled in this study received adjuvant CSPG4-antigen electrovaccination, according to different protocols. Clinical staging was performed by total body CT scan, alternatively three view chest radiographs and abdominal ultrasound were obtained. Local treatment consisted of \textit{en-bloc} surgical resection (maxillectomy, mandibulectomy, lip/cheek excision followed by reconstruction). The inclusion criteria were histological diagnosis of COMM surgically resected, availability of pre-treatment haematology with leukocyte differential count performed within 60 days before the surgery, absence of distant metastatic disease (M\textsubscript{1} of the TNM clinical staging system)\textsuperscript{31} and follow-up data of least 6 months. Dogs were excluded if, within 2-months before the definitive tumour treatment, they were
affected by a concomitant pathological condition able to compromise the minimum follow-up of 6 months and were administered with antibiotics and/or corticosteroids that could have interfered with NLR and LMR. For each patient the following parameters were recorded: signalment, tumour location, type of local treatment, TNM\textsuperscript{31} stage and outcome (cause of death and overall survival time). Overall survival time (OST) was defined from the day of surgery to the date of death or euthanasia, reporting if it was or not COMM-related. During the adjuvant vaccination protocol all the dogs were re-staged with radiographs or computed tomography. Given the different diagnostic accuracy of the two techniques in identifying pulmonary metastasis, only the overall survival time was chosen as end point in our statistical analysis. Follow up information was collected from medical records or by phone conversation with referring veterinarians and dog’s owners. Animals alive at the end of the study or lost to follow-up were censored at the date they were last known to be alive.

A written consent was obtained from the owners for the anesthetic, diagnostic, histological, and surgical procedures before staging. Regarding the adjuvant anti-CSPG4 DNA electrovaccination (started on 2009), dogs were treated according to the Good Clinical Practice guidelines for animal clinical studies. Both the Ethical Committee of the University of Turin and the Italian Ministry of Health approved the study; a written consent for entry into the study was obtained by dog owners.

Histopathology, blood collection and calculation of leukocyte ratios

For each biopsy specimen, degree of pigmentation (<50\% or \(\geq 50\) \% of pigmented cells), immunohistochemical analysis for Ki67 expression (using polyclonal Ki67 antibody A-047, DAKO; <19.5\% or \(\geq 19.5\)\%), mitotic index (MI, <4/10 high-power fields [hpf] or \(\geq 4/10\) [hpf]), nuclear atypia (<30 or \(\geq 30\) \% atypical nuclei in 200 cells counted), were evaluated.\textsuperscript{6,7} CSPG4 immunohistochemical score (from 0 to 8) was also available for all COMM samples.
and obtained as previously reported.\textsuperscript{32} Histologic evaluation of excision margins, bone invasion and presence of ulceration in the samples were also considered.\textsuperscript{33} An additional immunohistochemistry for CD3$^+$ lymphocytes was also performed on 10 samples randomly selected among COMM samples.

Pre-treatment haematological analysis on whole blood in ethylenediaminetetraacetic acid (EDTA) anti-coagulant, including complete blood count and leukocyte differentials, were performed by different laboratories. To verify instrumental results, May-Grunwald-Giemsa stain blood smears were assessed by clinical pathologists. Since different analysers were used, we considered universally accepted reference intervals\textsuperscript{34} as reported in Table 1. NLR and LMR were calculated as the ratio of the absolute count of neutrophils to lymphocytes and absolute counts of lymphocytes to monocytes, respectively. Regarding the clinical stage based on the TMN system,\textsuperscript{1,31} authors evaluated its impact on leukocyte ratios considering stage I-II (T$_{1,2}$, N$_0$, M$_0$) versus stage III-IV (Any T, any N, M$_0$). This stage division allowed to investigate lymph nodes involvement as a prognostic factor since none of our cases was classified as T$_3$N$_0$M$_0$ (stage III without lymph nodes involvement), consequently all dogs with COMM staged I-II were free of lymph nodes metastasis unlike dogs with COMM staged III-IV. Additionally, the impact of the T variable on NLR and LMR was carried out considering it individually (tumour size T$_1$ if $\leq$2cm, T$_2$ if between 2 and 4 cm, and T$_3$ if $>$4 cm).

\section*{Statistical Analysis}

Statistical analysis was applied to explore correlations between NLR and LMR with age, tumour pigmentation, T variable (tumour size), nuclear atypia, mitotic index, Ki67, CSPG4 status, ulceration, bone invasion, excision margin status, and clinical stage. Statistical analyses were performed with R, a free software environment for statistical computing and
graphics (version 3.6.3) and a P-value ≤0.05 was considered significant. Wilcoxon-Mann-Whitney rank sum test was used to evaluate NLR and LMR differences in classes based on stage/lymph nodes involvement (stage I-II vs stage III-IV), nuclear atypia, pigmentation, ulceration (presence/absence), margins (infiltrated vs not infiltrated) and bone invasion (presence/absence); Kruskal-Wallis rank sum test was applied to highlight differences in NLR and LMR values across subgroups based on the T variable (T1,2,3 based on TNM system); correlations between NLR and LMR with MI, Ki67, CSPG4, T and age (continuous variables) were evaluated by Spearman's rank correlation rho. The best cut off for NLR and LMR in terms of potential prognostic impact was estimated by cutpointr package.35 The median overall survival time was estimated by Kaplan Meier method and a log-rank test was used to compare the survival distribution of samples. Patients that died for causes different from COMM were censored from survival statistical analysis. Median time to follow up for the entire population was calculated from the day of surgery. After having obtained the best cut off for NLR and LMR, patients were divided into two classes above or under the cut off ratio in order to investigate a possible prognostic impact on survival. Additionally, Kaplan Meier curves were performed and settled considering three different end points of blood sample collection, in particular at 15, 30 and 60 preoperative days.

Results

Patients characteristic

A total of 86 dogs were retrieved from medical records, but only 39 dogs met all the inclusion criteria. The main reason of exclusion was unavailability of pre-treatment haematology with manual count within 2 months before definitive treatment. Median age was 11 years (range 8-14 years) and median weight was 25 kg (range 3-46 kg). Clinical
staging identified 6 COMMs staged I, 17 staged II, 14 staged III and 2 staged IV (M0).\textsuperscript{31}

According to the T variable of the TNM system, 20 COMMS were T\textsubscript{1}, 16 were T\textsubscript{2} and 3 were T\textsubscript{3}. Thirty-five dogs underwent definitive \textit{en bloc} excision of the primary tumour and regional lymphadenectomy, while four dogs had an excisional biopsy performed by the referring veterinarians.

At the end of the study 9 (23\%) dogs were still alive and 30 (77\%) were dead. Out of 30 dogs, 19 were dead because of COMM, in particular 4 for local recurrence, 13 for distant metastasis (lungs, brain) while 2 dogs were euthanized because of both. Eleven dogs succumbed for other causes: car accident (n=1), chronic kidney disease (n=3), neuro-orthopaedic disease (n=1), prostatic transitional cell carcinoma (n=1), laryngeal carcinoma (n=1), laryngeal paralysis (n=1), gastric ulcer (n=1) and 2 dogs for unknown reasons.

Median overall survival time was 943 days (range 68-2603 days, Figure 1). Median survival time for dogs that died for COMM was 412 days (range 68-972), while median survival time for dogs that died for causes other than COMM was 776 days (range 173-1768).

Median time to follow up for the entire population was 494 days (range 68-2603).

\textit{Histology and hematology}

Immunohistochemical scores for pigmentation, nuclear atypia, MI, Ki67 and CSPG4 are summarised in Table 2 and Table 3. Ki67 resulted undetectable in one case. Median MI was 12 (range 0-55), median tumour size was 2.5 cm (range 0.5-6). Twenty-one COMMs out of 39 presented ulceration. In two cases this information was not available. Excision surgical margins resulted histologically not infiltrated in 28 cases, infiltrated in 7 and not known in 4 cases. Histologically, not infiltrated margins exceeded 2 mm in all samples. On histology report bone invasion was detected in 22 out 39 COMMs. CD3\textsuperscript{+} immunohistochemical analysis detected only mild multifocal lymphocyte infiltration and inflammation in the 10
randomly chosen out of our 39 COMM samples; lymphocytes appeared more numerous in
the superficial corion (not associated with the tumour), being mostly localized within
ulcerated areas rather than being intratumoral.

Pre-treatment haematology analyses were performed after a median of 12 days (range 0-60
days) before the definitive treatment. Four dogs showed leukocytosis with neutrophilia, 2
further dogs revealed only neutrophilia, 3 dogs had lymphopenia, of which one with
monocytosis and one with monocytopenia. One dog displayed monocytosis. All these
changes were classified as mild, except in one case in which the neutrophilic leukocytosis
was marked. The hemograms of these patients are shown in Table 4. All the others 29 (74%)
patients exhibited values within normal limits. One dog was on medications for idiopathic
epilepsy while 3 further dogs had concurrent chronic renal concurrent disease.

Leukocyte ratios and cut off

Median NLR and LMR for all patients was 3.5 (range 1-14.2) and 3.8 (0.6-16), respectively.
NLR did not display association with the stage I-II (i.e., when there was no lymph node
involvement) vs. stage III-IV (i.e., COMMS with lymph node involvement) (p = 0.30) and
this occurred also for LMR (p = 0.21). No significant correlation was found between NLR
and LMR with histological parameters (pigmentation, nuclear atypia, ulceration, bone
invasion, Ki67, CSPG4 and MI), excision margin status (infiltrated vs. not infiltrated) and
age. Bivariate models with all correlation coefficients between histological parameters and
NLR and LMR are illustrated in Table 5 and Table 6, respectively. No statistically
significant differences among groups based on the T variable for NLR (p = 0.27) and LMR
(p = 0.11) were found. Through statistical software (cutpointr), an optimal NRL and LMR
cut off was identified in 2.792 and 3.622, respectively. Dividing all the patients into 4
groups with NLR > 2.792, NLR < 2.792, LMR > 3.622, LMR < 3.622, and comparing groups
with OST, the estimated cut off was not associated with survival (Figure 2). No statistical
correlation was found considering the entire population and survival time regardless NLR (p
= 0.22) and LMR (p = 0.92) cut off values, having blood samples collected within 60 days.
Lastly, no statistical correlation was found between leukocyte ratios and OST of dogs whose
blood sampling occurred at 15 days (NLR p = 0.67, LMR p = 0.38) and 30 days (NLR p =
0.66, LMR p = 0.61).

Discussion

This is the first report assessing the prognostic impact of pre-treatment NLR and LMR in
dogs with COMM and the first attempt to explore a possible correlation between NLR and
LMR with CSPG4 and well-known prognostic factors for COMM such as nuclear atypia,
pigmentation, Ki67, MI, clinical stage and lymph nodes metastasis. Considering the
explorative nature of the study also age, tumour ulceration, excision margin status and T
variable (tumour size) were included in the analysis.

In this sample population of dogs with COMM, pre-treatment NLR did not display a
prognostic impact on OST. This finding is in apparent contrast with previous studies
conducted on human melanoma in which the prognostic and predictive role of NRL has
been investigated over the last years. In a recent systematic review, an elevated NRL has
been recognized as a poor prognostic indicator in various types of cancer. Specifically, NLR
was elevated in patients with advanced disease evidenced by increased tumour stage, nodal
status and extent of the disease.

In the present study, the authors decided to consider the NLR and LMR rather than the
absolute neutrophils, lymphocytes and monocytes count in order to reduce the possible
variations of the single parameters. In fact, the number of neutrophils may change daily, being not always in line or proportional with lymphocytes. It has also been reported that NLR ratio can better reproduce fluctuations between neutrophils and lymphocytes.\textsuperscript{38}

The exact mechanism that binds elevated NLR and poor outcome in human melanoma patients remains unclear. Tumour promoting inflammation is one fundamental hallmark of cancer.\textsuperscript{19} In patients with advanced stage of cancer, the systemic inflammatory response leads to a change in blood cell composition that suggests an expansion of myeloid component (neutrophils and monocytes) caused by demargination and delayed apoptosis opposed to reduction of the lymphoid compartment as a result of margination and accelerated apoptosis.\textsuperscript{20} It seems that one of the tumorigenesis pathways mediated by neutrophils consist in the release of cytokines by melanoma cells that interface with receptors expressed on both neutrophils and melanoma cells through an autocrine effect.\textsuperscript{39}

Cytokines such as vascular endothelial growth factor (VEGF), tumour necrosis factor $\alpha$ (TNF-$\alpha$), interleukin-2 (IL-2), interleukin-6 (IL-6), and interleukin-10 (IL-10) act as inflammatory mediators providing an optimal tumour microenvironment for cancer progression.\textsuperscript{40} Indeed it has been widely recognised that tumour and its microenvironment direct neutrophils polarization and recruitment toward tumour tissue.\textsuperscript{41} Cancers related-neutrophils, circulating and tumours associated (TANs), are characterised by some functional plasticity. Thus, under the effect of cytokines, epigenetic modifications and microenvironment factors, neutrophils can acquire specific anti-tumour and pro-tumour functions and phenotypes. The first type of neutrophils (N1) exerts antitumour activity through direct and indirect cytotoxicity. The second type (N2) induces immunosuppression promoting tumour growth, angiogenesis and metastasis.\textsuperscript{41,42} A direct comparison between TANs and circulating neutrophils has not been assessed yet, probably due to the heterogeneous level of neutrophils’ infiltration in different cancers. However in a study performed in human patients with pancreatic cancer, the group with high NLR showed high
density of infiltrated macrophages and neutrophils compared with the low NRL group. At the moment, correlation between NLR and neutrophils tumour infiltration cannot be predicted but worth to be further investigated.

In this study 74% of our patients did not display alterations of their pre-treatment neutrophil and lymphocyte counts and this might explain why NLR did not show any prognostic significance.

In human oncology the strongest association between NLR and outcome has been found in cancers such as mesothelioma, where chronic inflammation covers a crucial role in tumour pathogenesis. A similar result was found by Chiti et al., in feline injection-site sarcoma, a tumour in which chronic inflammation plays a significant role as well. In that paper, NLR seemed to be significantly related to OST and disease-free interval in univariate analysis. On the human side, it has also been established that baseline NLR is strongly and independently associated with outcome of melanoma patients treated with ipilimumab. Specifically, it has been found that patients with NLR ≥3 had a significant risk of death and disease progression compared with those with lower NLR. Furthermore, in line with previous results, it has been found that, in humans with melanoma, a value of NLR ≥4 before starting ipilimumab treatment is associated with poor overall survival.

As for NLR, also the LMR has been widely investigated as prognostic tool in different types of solid cancer including metastatic melanoma corroborating the concept that a low LMR is associated with poor prognosis and shorter survival. Interestingly, a recent paper has examined the dynamics in the immune system in stage IV melanoma patients, obtaining serial concentration and measurements of cytokines and immune cell subtypes in peripheral blood. It has been found that LMR in many human melanoma patients had an oscillatory pattern and the progression free survival was significantly improved in those patients who received chemotherapy on the day LMR was elevated. Recent data suggest that, in
melanoma advanced patients, the systemic immunity undergoes to fluctuations and the immune system is oriented toward a state of inflammation where states of responsiveness are alternated to tolerance states. This aspect highlights that a single time-point measurement might be too limited to represent at the best the complex balance between tumour and immune system. Serial NLR and LMR values obtained from samples collected at different time points, closer to the day of first treatment, would probably better reflect the complex balance between the host’s pro-tumour neutrophils and the antitumour lymphocytes effect. In order to explore if blood sample time represents a relevant factor in terms of leukocytes variations and prognostic impact, we performed our statistical analysis on leukocytes ratio and OST selecting patients in which blood sample was collected at a different time point, i.e. at 15, 30 and 60 days; nevertheless no correlations were identified.

In the present study, no significant association of NLR and LMR with OST was observed. However, one aspect needs to be considered regarding patient selection. Most of human studies regarding leukocytes ratios have been performed, before and after the era of immune checkpoint inhibitors, in patients with advanced metastatic melanoma. However, some authors also investigated the prognostic relevance of peripheral blood cell count and their ratios in human melanoma patients at any stage, including early stage. They found that peripheral blood counts and ratios were not associated with survival of patients with localized or regionally metastasized melanoma; besides, blood cell counts were also similar among newly diagnosed patients and patients with recurrent or progressive disease. On disease progression from regional to distant metastatic disease (stage IV), NLR increased, LMR decreased and they were significantly associated with overall survival in multivariate analysis. All the dogs included in our study had evidence of metastasis, if any, not beyond the neck (M0), therefore involving only the draining lymph nodes of the neck (mandibular and/or retropharyngeal) and/or tonsil; this criterium has always been one of our inclusion parameters for immunotherapy. In line with this, it becomes important to remark that the
two dogs with COMM stage IV included in the study had only lymphatic bilateral metastasis at the level of the neck, therefore they were both T2N2bM0, according the WHO staging system.\textsuperscript{31} In this study neither the clinical stage nor lymph node involvement revealed an association with NLR and LMR. It is possible that a prognostic significance of NLR and LMR may be reached as soon as the distant metastatic progression develops, but not in the early stages, i.e. when COMM is still confined to the oral cavity and/or regional lymph nodes. Authors are absolutely aware of limitations that the present WHO TNM system implicate, which may lead to an imprecise staging of oral tumours. Besides, in this system the histological parameters are not taken into account and the tumour size is not proportioned to the size of the patient, thus leading to lack of standardization. For this reason, several histological parameters as well as the T variable were also included in the statistical analysis, substantiating the explorative aspect of the study. An additional critical issue in the present TNM system is the potential inaccuracy of the prognostic evaluation of clinical stage IV as this may include both dogs with (M1) or without distant metastasis (M0), including the latter dogs with bilateral lymphatic metastasis at the level of the neck only.

Regardless the treatment applied, median OST was 943 days (range 68-2383 days). This outcome is doubtless prolonged when compared with the previous outcomes reported for dogs with COMM.\textsuperscript{10} Authors could speculate that this positive outcome might be partially related to normal pre-treatment haematology analysis. However, it is important to consider that this positive outcome may also be related to the efficacy of the multimodality treatment (local treatment and immunotherapy) applied in this series of dogs.\textsuperscript{16}

No correlation was identified between NLR and LMR with known prognostic factors such as nuclear atypia, pigmentation, MI and Ki67. It is possible that these parameters do not or do minimally influence the leukocyte count in the same way as the outcome. The same consideration can be done about bone invasion and excision margin status despite their
recognized negative impact on prognosis.\textsuperscript{49,50} A further aspect that merits to be addressed is that an elevated T of the TNM system might be associated with a major risk of ulceration because of chronic trauma and possible inflammation; nevertheless, ulceration has not been identified as a prognosticator in oral melanoma\textsuperscript{7,49} and did not show any correlation with leukocytes ratios. In this study, those patients with ulcerated COMMS did not display any abnormalities on their haematology pre-treatment analysis (data not shown). The rationale behind performing CD3\textsuperscript{+} immunohistochemistry was an attempt to explore a possible correlation between local and systemic immune responses, triggered by tumour ulceration and inflammation, by comparing TILs and peripheral blood hematologic parameters not yet investigated in COMM. Interestingly, a relevance between TILs and hematologic parameters in breast cancer was demonstrated where LMR revealed significant correlation with CD8\textsuperscript{+}.\textsuperscript{51} However, a previous study has shown that, despite tumour infiltrating lymphocytes have been described in most melanocytic tumours, lymphocytic infiltration is usually mild.\textsuperscript{52} These data might be explained with high tumour mutational burden of melanoma and low expression of tumour-associated antigens by neoplastic melanocytes. Both factors could lead to a reduced immune stimulus for lymphocyte within the tumour and weak local anti-tumour immune response.\textsuperscript{53,54}

In this study no significant correlation was found between age and leukocytes ratios. In humans it is widely accepted that age represents an independent prognostic factor for melanoma specific survival.\textsuperscript{55} Furthermore, NLR has been found to correlate positively to age; this data is due to an upward trend of granulocytes count and lymphocyte count decline with age.\textsuperscript{56} In contrast with this, in our study more than 80\% of dogs older than 10 years had leukocytes counts within normal limits, but it should be outlined that this population of dogs is too small for make any assumption.
Finally, regarding CSPG4, a transmembrane glycoprotein expressed on melanoma cells and involved in tumour cell proliferation, migration and invasion,\(^{57}\) its immunohistochemical positivity ≥3 was not statistically correlated with OST.\(^{16}\) Even in this study, as previously reported,\(^{16}\) the CSPG4 score was not correlated with NRL and LMR, neither with OST.

This explorative study has several limitations; first of all, the number of patients is small to draw a final conclusion regarding NLR and LMR prognostic significance. Further limitations are the retrospective nature of the study and the lack of serial preoperative haematological evaluations. Therefore, the results obtained here should be considered cautiously. However, in our hands, pre-treatment NLR and LMR in dogs with COMM treated with surgery and immunotherapy were not associated with survivals when the evaluated hemograms preceded the definitive treatment. Nevertheless, as in human melanoma, it would be interesting to explore if there is any prognostic impact or correlation with survivals when patients develop distant metastasis. The true impact of NLR and LMR on the prognosis for oral canine MM needs to be better defined by further prospective studies involving a larger sample population.

References


### Table 1 Hemogram reference intervals

<table>
<thead>
<tr>
<th>Haematological value</th>
<th>References Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell count (WBCC)</td>
<td>6.0–17×10³/μL</td>
</tr>
<tr>
<td>Neutrophil count (NC)</td>
<td>3.0–11.5×10³/μL</td>
</tr>
<tr>
<td>Lymphocyte count (LC)</td>
<td>1.0–4.8×10³/μL</td>
</tr>
<tr>
<td>Monocyte count (MC)</td>
<td>0.15–1.35×10³/μL</td>
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</tbody>
</table>

### Table 2 Histological and immunohistochemical parameters of COMM from dogs included in the study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Threshold</th>
<th>Overall population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of pigmentation</td>
<td>&lt;50%</td>
<td>35 (90%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>≥50%</td>
<td>4 (10%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nuclear atypia</td>
<td>&lt;30%</td>
<td>12 (30%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>≥30%</td>
<td>27 (70%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mitotic index (MI)</td>
<td>&lt;4/10 hpf</td>
<td>8 (20%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>≥4/10 hpf</td>
<td>31 (80%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ki67</td>
<td>&lt;19.5%</td>
<td>11 (29%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>≥19.5%</td>
<td>27 (71%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>% in brackets

### Table 3 Immunohistochemical CSPG4 score of COMM from dogs included in the study

<table>
<thead>
<tr>
<th>CSPG4 score</th>
<th>patients n=39</th>
</tr>
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<tr>
<td>3/8</td>
<td>4</td>
</tr>
<tr>
<td>4/8</td>
<td>7</td>
</tr>
<tr>
<td>5/8</td>
<td>5</td>
</tr>
<tr>
<td>6/8</td>
<td>7</td>
</tr>
<tr>
<td>7/8</td>
<td>11</td>
</tr>
<tr>
<td>8/8</td>
<td>5</td>
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</tbody>
</table>
**Table 4** Patients with hemogram that underlined alterations

<table>
<thead>
<tr>
<th>Patient</th>
<th>WBCC (103/μL)</th>
<th>NC (103/μL)</th>
<th>LC (103/μL)</th>
<th>MC (103/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>15.3</td>
<td>11.8*</td>
<td>1.5</td>
<td>0.9</td>
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<tr>
<td>Patient 2</td>
<td>16.2</td>
<td>11.7*</td>
<td>3.1</td>
<td>0.6</td>
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<tr>
<td>Patient 3</td>
<td>11.5</td>
<td>9.4</td>
<td>0.8*</td>
<td>1.5*</td>
</tr>
<tr>
<td>Patient 4</td>
<td>6.8</td>
<td>5.5</td>
<td>0.8*</td>
<td>0.1*</td>
</tr>
<tr>
<td>Patient 5</td>
<td>33.1*</td>
<td>28.1*</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Patient 6</td>
<td>18.4*</td>
<td>14.5*</td>
<td>1.5</td>
<td>0.7</td>
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<tr>
<td>Patient 7</td>
<td>20.6*</td>
<td>16.2*</td>
<td>2.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Patient 8</td>
<td>18.7*</td>
<td>16.3*</td>
<td>1.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Patient 9</td>
<td>13.5</td>
<td>8.6</td>
<td>2.6</td>
<td>1.8*</td>
</tr>
<tr>
<td>Patient 10</td>
<td>8.5</td>
<td>6.5</td>
<td>0.9*</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* in bold values outside reference ranges.

**Table 5** Association between neutrophil to lymphocyte ratios (NLR) with stages, histological parameters and age in bivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>P-value</th>
<th>rho</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLR vs stage I-II/ stage III-IV</td>
<td>.30b</td>
<td>-</td>
</tr>
<tr>
<td>NLR vs pigmentation</td>
<td>.55b</td>
<td>-</td>
</tr>
<tr>
<td>NLR vs nuclear atypia</td>
<td>.54b</td>
<td>-</td>
</tr>
<tr>
<td>NLR vs ulceration</td>
<td>.71b</td>
<td>-</td>
</tr>
<tr>
<td>NLR vs margins (infiltrated/not infiltrated)</td>
<td>.29b</td>
<td>-</td>
</tr>
<tr>
<td>NLR vs bone invasion</td>
<td>.09b</td>
<td>-</td>
</tr>
<tr>
<td>NLR vs Ki67</td>
<td>.17</td>
<td>-0.22c</td>
</tr>
<tr>
<td>NLR vs CSPG4</td>
<td>.29</td>
<td>0.17c</td>
</tr>
<tr>
<td>NLR vs MI</td>
<td>.76</td>
<td>0.04c</td>
</tr>
<tr>
<td>NLR vs age</td>
<td>.48</td>
<td>-0.11c</td>
</tr>
<tr>
<td>NLR vs T (tumour size)</td>
<td>.97</td>
<td>0.05c</td>
</tr>
<tr>
<td>NLR vs T1,2,3</td>
<td>.27b</td>
<td>-</td>
</tr>
</tbody>
</table>

b Wilcoxon-Mann-Whitney rank sum test, Kruskall-Wallis rank sum test

c Spearman’s correlation coefficient
Table 6 Association between lymphocyte to monocyte ratio (LMR) with stages, histological parameters and age in bivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>P-value</th>
<th>rho</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMR vs stage I-II/ stage III-IV</td>
<td>.21\textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>LMR vs pigmentation</td>
<td>.76\textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>LMR vs nuclear atypia</td>
<td>.30\textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>LMR vs ulceration</td>
<td>.68\textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>LMR vs margins (infiltrated/not infiltrated)</td>
<td>.41\textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>LMR vs bone invasion</td>
<td>.09\textsuperscript{b}</td>
<td>-</td>
</tr>
<tr>
<td>LMR vs Ki67</td>
<td>.26</td>
<td>0.18\textsuperscript{c}</td>
</tr>
<tr>
<td>LMR vs CSPG4</td>
<td>.16</td>
<td>-0.22\textsuperscript{c}</td>
</tr>
<tr>
<td>LMR vs MI</td>
<td>.77</td>
<td>-0.04\textsuperscript{c}</td>
</tr>
<tr>
<td>LMR vs age</td>
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<td>0.14\textsuperscript{c}</td>
</tr>
<tr>
<td>LMR vs T</td>
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<td>0.20\textsuperscript{c}</td>
</tr>
<tr>
<td>LMR vs T\textsubscript{1,2,3}</td>
<td>.11\textsuperscript{b}</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{b} Wilcoxon-Mann-Whitney rank sum test, Kruskall-Wallis rank sum test
\textsuperscript{c} Spearman’s correlation coefficient

Captions to figures

Figure 1. Kaplan-Meier estimated survival probability of the entire population of dogs with COMM.

Figure 2. Kaplan-Meier curves considering NLR and LMR cut off. (A) Survival in days of dogs with COMM and a NLR cut off <2.792 (dotted lines) and NLR >2.792 (continuous line). (B) Survival in days of dogs with COMM and a LMR cut off <3.622 (dotted lines) and LMR >3.622 (continuous line).