Evaluation of leukocyte counts and neutrophil-to-lymphocyte ratio as predictors of local recurrence of feline injection site sarcoma after curative intent surgery

EVALUATION OF LEUKOCYTE COUNTS AND NEUTROPHIL-TO-LYMPHOCYTE RATIO AS PREDICTORS OF LOCAL RECURRENCE OF FELINE INJECTION SITE SARCOMA AFTER CURATIVE INTENT SURGERY

Abstract

Local recurrence (LR) is the major concern in the treatment of feline injection-site sarcoma (FISS). Pretreatment leukocyte counts and ratios have been reported as diagnostic and/or prognostic markers in human and canine oncology. The aim of this retrospective study was to explore the prognostic impact on LR and overall survival time (OST) of pretreatment neutrophil-to-lymphocyte ratio (NLR), white blood cell count (WBCC), neutrophil count (NC) and lymphocyte count (LC) in cats with surgically excised FISS.

Eighty-two cats with histologically confirmed FISS at first presentation, without distant metastases, and with available pretreatment hematological analyses were retrospectively enrolled. The correlation of NLR, WBCC, NC, LC with tumor variables and patient variables was explored. NLR was correlated with tumor size (*P* 0.004), histological pattern of tumor growth (*P* 0.024) and histotype (*P* 0.029), while WBCC and NC were associated with ulceration (*P* 0.007, *P* 0.011) and pattern of growth (*P* 0.028, *P* 0.004). No significant relationships emerged between LC and any of the considered variables. The impact of NLR, WBCC, NC, LC on LR and OST was then estimated in univariate and multivariate analysis. In univariate analysis, NLR, WBCC, NC were significant prognostic factors for both LR and OST. NLR, WBCC, NC remained prognostic in multivariate analysis for LR but not for OST. When NLR, WBCC and NC were jointly analyzed, WBCC was the marker with the greater impact on LR. Preoperative NLR, WBCC, and NC may aid in identifying cats at higher risk of LR.

Introduction

Feline injection-site sarcoma (FISS) is among the four most common feline skin cancers.[1] Although it was initially hypothesized that the etiology of FISS is strictly related to vaccination[2], it is nowadays widely accepted that this tumor can develop following any stimulus that causes chronic local inflammation of the subcutis or muscles.[3-7] Despite the relatively low incidence of distant metastasis (0–28%)[8-11], FISS tends to be locally aggressive and local recurrence (LR) represents the major concern, with reported rates as high as 14%–42%;[8,12-15] therefore, achieving adequate control of local disease through wide-margin/radical surgical excision is the cornerstone in oncological management of the tumor[14,16].

The prognostic impact of different variables on LR and overall survival time (OST) after wide-margin/radical excision has been widely explored in the last two decades. Histological grading is considered one of the most important prognostic factors for canine soft tissue sarcomas[17] (STS); nonetheless, the traditional three-tier grading system has shown limited value in predicting LR and outcome for FISS.[11,13,17-18]

Completeness of excision is indeed crucial in obtaining long-term control of the local disease; however, LR has been reported even in the presence of histologically non-infiltrated margins, suggesting that factors other than completeness of excision may be implicated in tumor relapse.[11,13,19]

Although other variables, such as size and location of the tumor, expertise of the surgeon, recurrent tumors, p53 expression, mitotic count, and differentiation, have been proposed as predictors of LR, there is still no consensus on which factors other than completeness of excision should be taken into account to predict prognosis.[8,13,15,20,21] Hence, readily available and accurate variables that can aid in identifying cats at higher risk of LR are desirable to improve oncological management of these patients.

Pretreatment neutrophil-to-lymphocyte ratio (NLR) is a marker of systemic inflammatory response that has been reported to be a useful prognostic tool for several solid tumors in human medicine, including STS.[22] Recently, the veterinary literature has reflected growing interest in peripheral blood cell abnormalities as diagnostic and prognostic markers for both neoplastic and inflammatory conditions. A few papers have explored the prevalence of different leukocyte populations and T-lymphocyte subsets in tumor-bearing versus healthy dogs.[23-26] Furthermore, leukocyte counts and ratios have been proposed as diagnostic and prognostic tools for dogs with lymphoma, osteosarcoma, mast cell tumors, and STS.[27-31] Yet, the prognostic impact of peripheral leukocyte

counts and ratios, including NLR, has not been assessed in feline medicine. Given the promising results described in canine oncology, this retrospective study aimed to explore the prognostic impact on LR of pretreatment white blood cell count (WBCC), neutrophil count (NC), lymphocyte count (LC), and NLR in cats with newly diagnosed, surgically excised FISS. As a secondary aim, the impact of leukocyte counts and NLR on OST was assessed.

Materials and Methods

Records (January 2002 to December 2017) from the XX and YY were searched for client-owned cats with histologically confirmed FISS. The main inclusion criteria were: FISS at first presentation, absence of distant metastasis (assessed by total body contrast-enhanced CT or thoracic radiography and abdominal ultrasound), and treatment by wide-margin/radical surgical excision with three to five cm lateral margins and two deep fascial planes or limb or tail amputation. To be eligible for inclusion, presurgical complete hematological data with leukocyte differential (within 45 days before surgery) had to be available. Exclusion criteria were: (1) neoadjuvant chemotherapy and/or radiotherapy, (2) adjuvant radiotherapy, (3) FIV and/or FeLV positivity, (4) administration of antibiotics and/or corticosteroids within two months before surgery, and (5) lack of availability of postoperative follow-up information.

Data retrieved from the medical records of included cats were: signalment (breed, sex, age, weight), characteristics of the primary tumor (location, size at clinical examination, ulceration), presurgical laboratory data (leukocyte counts, hyperglycemia as indicator of stress), concomitant diseases if present, histological findings (histotype, necrosis, grading, pattern of growth, margin status), and outcome (time to LR, time to metastases, cause of death, OST).

For pretreatment hematological analysis, blood was collected in EDTA, and CBC parameters, including leukocyte differential, were measured with the same laser-based analyzer at the laboratory of one of the two institutions (ADVIA®120 Hematology System, Siemens Diagnostics). Instrumental differential was also checked microscopically on May-Grunwal-Giemsa stained smears. NLR was calculated as the ratio of the absolute count of neutrophils to lymphocytes. When the biochemistry profile was available, hyperglycemia was retrieved and considered as a possible marker of stress that could have altered the absolute values of both neutrophils and lymphocytes and, as a consequence, the NLR. Variations from normal values of WBCC, NC, and LC were defined based on the reference intervals reported in Table 1.

Histopathological specimens were processed and examined by the same pathologist at each institution (XX, XY). Variables retrieved from the histological reports were: histotype, pattern of growth[32] , necrosis (semiquantitatively scored as 0 when absent, 1 when <25%, 2 when 25%-50%, and 3 when >50%), histological grading[17] if available, and status of surgical margins[13] (infiltrated

or non-infiltrated). Surgical margins were evaluated combining, at trimming, two techniques: radial sectioning along the longest axis of the sample (perpendicular margins)[33], and tangential (*en face*) sectioning/3D technique[13,33].

Follow-up information was obtained from the clinical records or by telephone conversations with cat owners or referring veterinarians. Time to LR and OST were the primary and secondary endpoints, respectively. Time to LR was calculated from the date of surgery to the date of LR, defined as a cytologically or histologically confirmed FISS growing within two cm from the scar of previous excision. OST was calculated from the date of surgery to the date of death or euthanasia; cause of death was classified as either tumor related or tumor unrelated.

Statistical analysis

Statistical analysis evaluated the relationship between NLR, WBCC, NC, and LC and the following variables: age, glycemia, tumor size, ulceration, concomitant diseases, tumor necrosis, tumor histotype, and histological pattern of growth. The impact of NLR, WBCC, NC, and LC on LR and OST was then assessed.

Pearson coefficient (*r*) was used to assess the linear correlation between NLR, WBCC, NC, and LC and continuous variables (tumor size, glycemia, age). Results were reported as estimated correlation coefficient and 95% confidence intervals (CI). The strength of the correlation was interpreted following the rule of thumb.[34] The null hypothesis of the absence of correlation between two variables was tested by *t* statistics.

For categorical variables (ulceration, concomitant diseases, necrosis, histotype, pattern of growth), the distributions of NLR, WBCC, NC, and LC for each modality were reported as: minimum, Q1 (25%), mean, median, Q3 (75%), and maximum. As NLR, WBCC, NC, and LC were non-normally distributed, non-parametric tests were used to compare the distributions of the above-mentioned variables in the classes of categorical variables. Wilcoxon rank sum test with continuity correction was used for the variables with two modalities and Kruskal-Wallis test for the variables with three modalities.

The probability of being free of LR during follow-up was estimated by the Kaplan-Meier method. For cats that died without developing LR, times to death were censored to the death date, assuming independence between time to LR and time to death without LR. Patients lost to follow-up were censored at the time of the last contact. However, since the above cited independence assumption could not be determined on the basis of the available follow-up data, we also reported the bounds in which the correct estimates of LR free survival it is expected to lie, avoiding the assumption of independence.[35]

The probability of surviving during follow-up was estimated by the Kaplan-Meier method. Cox regression model was used to explore the prognostic impact of NLR, WBC, NC, LC and all other examined clinical and pathological variables on LR and OST. Firstly, univariate analysis was performed; then, a multivariate model was used to evaluate the prognostic role of each hematological value, adjusted for the clinical and pathological variables which resulted statistically significant in univariate analysis. For OST analysis, the maximum number of variables that could be included in the model was determined following the EPV rule.[36] For LR analysis, a less conservative rule[37] was applied, thus results of this analysis should be considered preliminary. A backward selection procedure was used to obtain a final model that included only statistically significant variables. To assess the robustness of multivariate analysis, bivariate models were also performed adjusting NLR, WBCC, NC for each one of the clinical and pathological variables. Categorical variables were included into the model as dummy variables, while continuous variables were included in their original measurement scale. For these latter, the possible non-linear relationship, was evaluated by regression cubic splines and the contribution of non-linear terms was tested by the likelihood ratio test. Results were reported as cause-specific hazard ratios for LR and hazard ratios for OST, and 95% CI. The Wald test was used to assess the significance of the regression coefficients of the model. No competing risks were considered for LR, as this event was recorded regardless of the occurrence of distant metastases. Predictive accuracy of the survival model was determined by calculating the area under the curve (AUC) extended for survival analysis (Harrell C statistic). Time-dependent ROC curves were used to find the best cut-off values for NLR, WBCC, and NC on the basis of the Youden Index (i.e. sensitivity + specificity).[38]

Median follow-up time was estimated with the reverse Kaplan-Meier method.[39]

All statistical analyses were performed with a software package (R-Software; www.r-project.org) and a P value ≤ 0.05 was considered significant.

Results

Eighty-two cats fulfilled the inclusion criteria of the study, including 41 neutered females, 38 castrated males, two intact females, and one intact male. Cat breeds included 71 domestic shorthairs, three Persians, three Carthusians, two Siamese, two Norwegians, and one Maine Coon. Median age at presentation was 11 years (range 6–18 years), and median weight was 4.5 kg (range 2.5–8.5 kg).

Tumor size at the longest diameter was available in 81 cats, and median tumor diameter was four cm (range 0.7–15 cm). Tumor location was interscapular in 53 (64%), thoracic wall in 17 (21%),

abdominal wall in 8 (10%), and tail and limbs in 4 (5%). In 8 cats (10%) the tumor was ulcerated at presentation.

Pretreatment hematology analyses were performed a median of 16.5 days before surgery (range 1 – 45 days) and revealed leukocytosis in 6 (7%) cats, leukopenia in 22 (27%), neutrophilia in 6 (7%), neutropenia in 12 (15%), and lymphopenia in 48 (59%); 29 patients (35%) had leukocyte values within the normal ranges. Glycemia was available for 78 patients (95%), of which 23 (30%) were hyperglycemic and 46 (59%) normoglycemic.

Concomitant diseases were recorded in 25 cats (30%) at the time of surgical consultation and included: chronic kidney disease (n=9), hyperthyroidism (n=4), chronic rhinitis (n=2), urinary tract infection (n=2), cardiac hypertrophy (n=2), benign keratin cyst (n=1), gastoenteropathy (n=2), diabetes (n=1), and allergic dermatitis (n=2).

Total body contrast-enhanced CT was performed in all but three patients that had thoracic radiographs and abdominal ultrasound. Seventy cats (85%) were treated with surgery alone, and 12 (15%) received adjuvant chemotherapy (Doxorubicin 1 mg/kg IV every 3 weeks for 4 cycles in 6 cats; Carboplatin 200 mg/m^2 IV every 3 weeks for 4 cycles in 6 cats).

Histopathological report described 52 fibrosarcomas (64%), 11 pleomorphic sarcomas (13%), and 19 malignant fibrous histiocytomas (23%). Pattern of growth was available in 79 cases and was considered expansile in 36 tumors (46%) and infiltrative in 43 (54%). Necrosis was scored 0 in 15 tumors (18%), 1 in 8 (10%), 2 in 29 (35%), and 3 in 28 (34%); in 2 cases the percentage of necrosis was not reported. Histological grading was available in 62 reports: 9 tumors were classified as grade I (15%), 26 as grade II (42%), and 27 as grade III (43%). Surgical margins were non-infiltrated in 65 specimens (79%) and infiltrated in 17 (21%).

At the end of the study, ten cats were alive without signs of local and/or distant relapse, 14 were lost to follow-up, and 58 were dead, including 24 that died of tumor-related causes and 34 that died of causes other than FISS, including chronic kidney disease (n=16), hypertrophic cardiomyopathy (n=3), intestinal lymphoma (n=4), car accident (n=3), acute pancreatitis (n=2), oral squamous cell carcinoma (n=2), recurrent urethral obstruction (n=2), meningioma (n=1), transitional cell carcinoma of the urinary bladder (n=1). Of the 24 cats that died because of FISS, 17 had a LR, three had LR and pulmonary metastasis, and the remaining four cats had pulmonary metastasis alone. Median time to LR was not reached (Figure 1). The estimates reported in Figure 1 are based on the assumption of independence between time to relapse and time to death, which could nott be evaluated. However, it is possible to obtain intervals (bounding) into which the estimates are expected to lie if the assumption were not tenable. For example, at 550 and 1100 days the Kaplan-

Maier estimated probability of being free from LR is 78% and 70% respectively, with a corresponding bounding of 57-81% and 43-78%.

Median OST was 975 days (Figure 2). Median follow-up time was 1790 days.

White blood cell count, neutrophil count, lymphocyte count and neutrophil-to-lymphocyte ratio

WBCC and NC were significantly higher in ulcerated tumors (*P* 0.007; *P* 0.011). WBCC, NC and NLR were significantly higher in histologically infiltrative FISS (*P* 0.028; *P* 0.004; *P* 0.024). (Tables 2 and 3). NLR was also significantly higher in fibrosarcomas (*P* 0.029) and was correlated with tumor size (r 0.3215; *P* 0.004). None of the tested variables showed a relationship with LC (Tables 2 and 3).

In univariate analysis, a statistically significant prognostic impact on LR was found for WBCC (*P* 0.003), NC (*P* 0.003), NLR (*P* 0.015) but not for LC. The hazard of LR increased with increasing values of WBCC, NC and NLR (Table 4). With regards to diagnostic accuracy, for WBCC AUC was 0.695 at one year, 0.614 at two years and 0.599 at three years, with best estimated cut-off of 10.270 ($\times 10^3/\mu L$) at one year (sensitivity=0.543; specificity=0.777), 11.240 ($\times 10^3/\mu L$) at two years (sensitivity=0.380; specificity=0.827), and 11.460 ($\times 10^3/\mu L$) at three years (sensitivity=0.287; specificity=0.840). For NC, AUC was 0.731 at one year, 0.653 at two years and 0.664 at three years, with best estimated cut-off of 4.960 ($\times 10^3/\mu L$) at one year (sensitivity=0.831; specificity=0.542) and two years (sensitivity=0.695; specificity=0.523), and 6.940 ($\times 10^3/\mu L$) at three years (sensitivity=0.482; specificity=0.791). For NLR, AUC was 0.630 at one year, 0.568 at two years, and 0.585 at three years. The optimal cut-off value for NC to predict LR at one year was 1.823, which yielded a sensitivity of 0.947 and a specificity of 0.296; the optimal estimated cut-off at two and three years was 3.654 (sensitivity=0.525; specificity=0.660) and 3.654 (sensitivity=0.523; specificity=0.669).

WBCC (*P* 0.011), NC (*P* 0.014) and NLR (*P* 0.028) were prognostic for OST as well, with increasing hazard of death for increasing values of WBCC, NC and NLR (Table 4). LC was not prognostic for OST.

Of the examined clinical and pathological variables, concomitant diseases, ulceration, histological pattern of growth and margins status were prognostic in univariate analysis for LR, while age, ulceration, tumor size, and margins status were prognostic for OST (Tab 4).

In multivariate analysis, WBCC (*P* 0.003), NC (*P* 0.004) and NLR (*P* 0.016) remained independently prognostic for LR (Table 5). However, when WBCC, NC and NLR were considered together in Cox regression model, only WBCC remained prognostic for LR (*P* 0.012). Concomitant diseases, ulceration, and margin status remained prognostic as well, while pattern of growth

(expansile VS infiltrative) was removed from the model by backward selection procedure because it was no longer statistically significant (Table 5). Bivariate models confirmed the results of the multivariate analysis.

For OST, none of the clinical/pathological variables that were significant in univariate analysis (age, ulceration, tumor size and margins) was removed from the multivariate model by the backward procedure. WBCC, NC and NLR were not confirmed to be prognostic for OST in the multivariate model. Age, ulceration, margins and tumor size remained prognostic (Table 6).

Discussion

In the study population, pretreatment NLR, WBCC, and NC had a prognostic impact in univariate analysis on both LR and OST in cats with FISS at first presentation that underwent curative-intent surgery. LC, conversely, was not useful in predicting LR or OST. However, NLR, WBCC and NC were not confirmed to be prognostic for OST in the multivariate model, considering other clinical-pathological variables.

Surprisingly, when WBCC, NC, NLR were considered together in the Cox regression model, only WBCC remained prognostic for LR, while NLR and NC lost their significance. This finding is in contrast with previous reports in human and canine oncology.[22,27,31] Indeed, NLR and other leukocyte ratios reportedly have higher prognostic impact than leukocyte counts due to their greater stability and lower susceptibility to fluctuations of single cell populations caused by pathophysiological changes.[27,31] Since NLR takes into account both the impact of variations of NC and LC, the fact that NLR was inferior in predicting LR may be due to the lack of significance of LC; inclusion of this variable in the ratio may have reduced the prognostic significance of NLR. The fact that, despite having a higher proportion of lymphopenic rather than neutrophilic cats in the study population, LC was not correlated with LR nor OST, further corroborates this consideration. Similarly, as neutrophils constitute the majority of WBC in cats, the higher impact of WBCC compared with NLR may simply reflect the higher contribution of NC to this value. A recent paper evaluated NC and morphology in 517 cats with various diseases and reported a higher mortality for neutrophilic patients.[40] Although it is difficult to extrapolate whether oncological cats were included in the study, this finding corroborates the role of neutrophilic immune response in the feline species.[40] Nonetheless, in light of these considerations, it is surprising that NC lost its significance as well; however, this result should be considered cautiously, and further studies on a wider sample size are warranted to confirm the superiority of WBCC in predicting LR in FISS.

Among the examined tumor variables, pattern of tumor growth was the only one that showed a significant correlation with all the hematological variables in univariate analysis; indeed, patients with infiltrative tumors tended to have higher values of pretreatment WBCC, NC, and NLR. This result may suggest a relationship between pattern of tumor growth and the clinical behavior of FISS, with invasive tumors showing a more aggressive behavior that triggers an immune response with higher values of WBCC, NC, and NLR. Although histological pattern of growth has not been previously studied in FISS, this finding is consistent with a previous report on canine STS, where histologically invasive variants were associated with higher recurrence rates. [32] However, this consideration should be considered with caution, as in multivariate analysis tumor pattern of growth was not prognostic for LR, and the prognostic impact of this variables should be further investigated in future studies.

While NLR did not show any correlation with tumor ulceration, both NC and WBCC were associated with such variable. It might be argued that the better predictive accuracy of the leukocyte count is due to its correlation with tumor ulceration, which is a well-known prognostic factor for other solid tumors[41]; however, even though in our study ulceration was prognostic for both LC and OST, its significance in FISS should be confirmed in further studies in order to accept or refuse this hypothesis.

Other histological and clinical tumor variables were inconsistently correlated with the above-mentioned parameters, with presence of ulceration influencing WBCC and NC but not NLR, and tumor histotype and size influencing NLR. Tumor necrosis was not correlated with any of the above-mentioned hematological parameters, perhaps because necrosis remains circumscribed within the tumor pseudo capsule, that may hide it to the immunity system, thus precluding an immune response against it.

In the present study, we proposed different cut-offs for NLR, WBCC, and NC for prediction of LR at one, two, and three years, as the different follow-up times of the included patients precluded the determination of a single cut-off value. However, since the majority of events happened during the first year of follow-up, the cut-off at one year should be considered the most reliable, as suggested by the higher sensitivity and specificity of this value compared with the cut-offs at two and three years. These values should be considered barely preliminary, and more reliable cut-offs need to be determined in prospective studies on a larger population in order to allow validation on an independent sample.

The estimated cut-offs for leukocyte counts in our study population fall in their physiological ranges. However, it should be noticed that in the study design it was decided to consider leukocyte counts as continuous rather that categorical variables, and this decision was mainly due to the

306 explorative nature of the study. In fact, since to the authors' knowledge no previous papers have
307 examined the significance of leukocyte fluctuations in feline oncology, it would have been
308 misleading to establish empiric cut-offs. As a consequence, our results and the estimated cut-offs on
309 the present study population suggest that for each increase in the WBCC and NC determines an
310 increase in the hazard of LR, regardless of absolute alterations in the physiological ranges of
311 leukocyte counts.
312 Cut-off values for NLR, WBCC, and NC were estimated for the prediction of LR but not OST. LR
313 was a measurable outcome, even in the retrospective setting of this report, as cytological or
314 histological biopsies were always performed to confirm it; on the other hand, OST may have been
315 influenced by the decision of the owners to euthanize their cat and was thus considered a less
316 reliable endpoint.
317 Which variables should be considered when planning treatment and predicting prognosis in FISS is
318 an open debate; however, several papers have shown that the recurrence rate is significantly lower
319 for cats with histologically non-infiltrated margins versus infiltrated margins, and completeness of
320 excision has been reported to be a prognostic factor for survival.[11,13,15] Consistently with previous
321 studies, margin status resulted prognostic for both LR and OST in our report. In the bivariate model
322 that was performed as a more robust analysis to confirm the results of the multivariate model, the
323 prognostic impact of margin status on both LR and OST was confirmed. Furthermore, even though
324 our primary aim was to evaluate the prognostic impact of leukocyte counts and NLR on LR and
325 OST, other potentially prognostic clinical and pathological variables were included in the survival
326 analysis. Ulceration resulted significantly prognostic for both LR and OST. Although ulceration has
327 not been previously reported to be prognostic for other canine or feline soft tissue sarcomas, it is
328 considered as negative prognostic factor for other solid tumors, such as canine mast cell tumors [41.] It
329 may thus be hypothesized that this characteristic is correlated with a more aggressive clinical
330 behavior that causes a rapid tumor growth and subsequent disruption of the cutis.
331 Other clinical-pathological variables that had a prognostic impact were concomitant diseases for
332 LR, and age and tumor size for OST; however, given the low numerosity of events in this study
333 population, it would be speculative to draw conclusions from these results, especially considering
334 that such variables resulted prognostic for only one of the considered end-points. Further studies are
335 thus needed to assess the real impact of such variables on LR and OST.
336 To the authors' knowledge, this is the first report to evaluate the prognostic value of leukocyte
337 counts and ratios in tumor-bearing cats. The prognostic impact of different leukocyte populations,
338 however, has previously been assessed in both human and canine oncology. In human medicine,
339 higher values of pretreatment NLR have been correlated with poorer prognosis for several solid

malignancies, including STS.[22] The prognostic/diagnostic value of different leukocyte populations, such as neutrophils, lymphocytes, eosinophils, and monocytes, and their ratios has been explored in canine solid and lymphoproliferative neoplasms.[26-31,42] In a recent paper, NLR was significantly higher in dogs with STS than in those with benign soft tissue lesions, although the parameter was not predictive of tumor grade.[30] In two further studies focusing on canine mast cell tumor, higher NLR was predictive of higher tumor grade and was correlated with poorer outcome in univariate analysis, although it was not confirmed as an independent prognostic factor for survival in multivariate analysis.[29,31] Neutrophilia has also been linked with tumor-associated systemic inflammatory response for different canine malignancies, such as oropharyngeal cancer and acute leukemia.[24,42] In a paper evaluating the prevalence of leukocyte populations in healthy, older, and tumor-bearing dogs, the authors reported that tumor-bearing dogs had a higher number of WBC and a higher percentage of neutrophils.[43] Such findings are consistent with the results in the present study.

The mechanism responsible for the relationship between leukocyte counts and ratio alterations and prognosis in human and veterinary oncology remains unclear. Indeed, tumor microenvironment and type of host immune response against cancer play a role in tumor development and progression. Neutrophils have both pro- and antitumor activities, but in cancer patients, neutrophilia has been linked with angiogenesis and tumor progression. Moreover, granulocytes and granulocyte-macrophage colony-stimulating factors are produced by some solid tumors and are known to modulate their progression in people[44-45]. On the other hand, T-lymphocyte subsets are crucial in specific antitumor immunity, and lymphopenia has been correlated with a worse prognosis in human patients with solid tumors.[46] As a consequence, a high NLR may reflect a less effective immune response against cancer, with a predominant neutrophilic response and relative lymphopenia, which promotes tumor growth and dissemination.[23,43,47]

In the light of such considerations, the absence of a correlation between LC and both LR and OST in the sampled population is surprising; however, it may be hypothesized that the efficacy of the host immune response against cancer is more influenced by the subpopulations of lymphocytes than by the absolute lymphocyte count, as suggested by results of previous studies on dogs.[23,25,43]

This report has several limitations, mainly related to its retrospective nature. The relatively low number of included cats resulted in a low number of events (24/82), which compromised the robustness of multivariate analysis. This consideration holds particularly true for multivariate analysis on LR, where the number of variables to be included in the model was decided following a less restrictive rule than the traditional EPV rule. However, a more robust bivariate model was performed and confirmed the results of multivariate analysis. Furthermore, when the relationship

between NLR, WBCC, NC, and LC and factors that may have altered leukocyte populations was explored, age, presence of concomitant diseases, and hyperglycemia were not associated with any of the above-mentioned hematology values, suggesting that such variables are unlikely to have an impact on them.

The decision to include cats that received adjuvant chemotherapy was mainly dictated by the fact that, to date, there is no strong evidence that such treatment is effective against LR, and it would thus have been unlikely to influence this endpoint.[48-51] In the sampled population, (4/6) 30% of cats treated with chemotherapy developed LR, while 18/76 (24%) of the cats that received only surgery reached this endpoint, suggesting a lack of impact of this treatment against LR. It might be argued that adjuvant chemotherapy may prevent distant metastasis, thus influencing OST, which was the secondary endpoint of the study; however, of the 24 cats that died due to tumor-related causes, only two had distant relapse, while the remaining 22 all experienced LR, suggesting that the impact of distant metastases on OST is negligible. Finally, in univariate analysis adjuvant chemotherapy was not significant for neither of the endpoints, suggesting the low impact of such treatment on outcome.

Strengths of this report are the homogeneity of patient management and long-term follow-up. Indeed, all cats were routinely staged, with most patients receiving a preoperative total body CT, definitive treatment consisted of curative-intent surgery for all patients, while cats that underwent neoadjuvant treatments were excluded; furthermore, we excluded patients that received radiotherapy as such treatment is reportedly effective against tumor recurrence.[12,50,52] Finally, the median follow-up of 1790 days allowed for a reliable observation of LR, including long-term recurrences.

In conclusion, pretreatment NLR, WBCC, and NC may be of value in identifying cats at higher risk of LR after curative-intent surgery for FISS. NLR, WBCC, and NC are readily available, cost-effective, and objective prognostic tools that can be easily retrieved from routine preoperative hematological work-up without the need for invasive examinations or adjunctive cost for the owners. However, the exploratory nature of this study impeded the identification of reliable thresholds, and further prospective studies are warranted to confirm the prognostic impact of these parameters on surgically excised FISS and to determine more accurate cut-offs.

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Tables

Table 1. References intervals for WBC, NC, LC in the cat.

Hematological parameter	**Reference Interval**
White Blood Cells (X $10^3/\mu L$)	6.0 - 17
Neutrophil Count (X $10^3/\mu L$)	3 – 13.4
Lymphocyte Count (X $10^3/\mu L$)	2 – 7.2

Table 2. Comparison of Neutrophil/Lymphocyte ratio (NLR), Absolute Neutrophil Count (NC), White Blood Cell Count (WBCC), †Lymphocyte Count (LC) distribution in the categories of ulceration, necrosis, histology, concomitant disease and margins.

Variable	**median,(Q1,Q3)**	***Mean(s.d.)***	***P***
NLR *vs* **Ulceration**[§] Absent Present	 3.069(1.756, 4.730) 3.199(2.555, 9.736)	 4.952(6.547) 7.476(8.009)	0.226
NC *vs* **Ulceration**[§] Absent present	 3.482 (4.765, 6.970) 6.052 (7.250, 10.820)	 6.095(4.191) 12.730(14.007)	0.011*
WBC *vs* **Ulceration**[§] Absent Present	 7.260(5.467, 10.400) 10.680(9.018, 14.900)	 8.680(4.822) 16.010(13.880)	0.007*
LC *vs* **Ulceration**[§] Absent Present	 1.565(1.002, 2.530 2.120(1.720, 2.425)	 1.877(1.140) 2.056(0.713)	0.407
NLR *vs* **Expansile/Infiltrative**[§] Infiltrative Expansile	 3.405(2.331, 6.359) 2.444(1.594, 3.749)	 6.695(8.531) 3.536(3.742)	0.024*

NC *vs* **Expansile/Infiltrative**§ Infiltrative Expansile	4.230(5.880, 8.410) 3.102(4.040, 5.628)	8.311(7.768) 5.092(2.957)	0.004*
WBC*vs* **Expansile/Infiltrative**§ Infiltrative Expansile	8.620(6.66, 11.53) 7.025(5.245, 9.310)	10.98(8.171) 7.800(3.707)	0.028*
LC *vs* **Expansile/Infiltrative**§ Infiltrative Expansile	1.690(1.080, 2.420) 1.775(1.182, 2.688)	1.875(1.194) 1.986(1.015)	0.472
NLR *vs* **Necrosis**¶ 0 1 2 3	 3.280(1.760, 4.770) 2.281(1.762, 3.488) 2.696(1.680, 4.374) 3.628(2.186, 7.356)	 5.458(6.148) 2.671(1.232) 3.616(2.689) 7.243(9.827)	0.401
NC *vs* **Necrosis**¶ 0 1 2 3	 3.895(4.700, 7.450) 3.325 (4.165, 5.690) 3.810 (4.710, 6.200) 3.710 (5.550, 9.808)	 6.579(4.170) 4.492(1.709) 5.654(3.316) 8.524(9.077)	0.498
WBC *vs* **Necrosis**¶ 0 1 2 3	 8.100(6.295, 10.200) 6.200(5.072, 7.650) 7.240(6.020, 9.900) 8.805(5.872, 13.260)	 9.255(4.374) 6.726(2.278) 8.536(4.706) 11.130(9.301)	0.360
LC *vs* **Necrosis**¶ 0 1 2 3	 2.030(0.980, 2.840) 1.785(1.495, 2.320) 1.710(1.240, 2.540) 1.465(1.062, 2.455)	 1.931(0.993) 1.895(0.827) 2.100(1.372) 1.719(0.9425	0.799
NLR *vs* **Histotype**§ Fibrosarcoma other	 2.619(1.705, 4.110) 3.752(2.347, 7.674)	 4.682(7.130) 6.092(5.860)	0.029*
NC *vs* **Histotype**§ Fibrosarcoma other	 3.385 (4.765, 6.920) 4.358 (5.535, 9.962)	 5.674(3.376) 8.594(8.769)	0.104
WBC *vs* **Histotype**§ Fibrosarcoma other	 7.135(5.542, 10.170) 8.845(6.050, 11.730)	 8.368(4.130) 11.170(9.123)	0.187
LC *vs* **Histotype**§ Fibrosarcoma Other	 0.980(1.855, 2.630) 1.105(1.515, 2.258)	 1.985(1.193) 1.737(0.927)	0.528
NLR *vs* **Concomitant**			0.600

disease[§] Absent Present	3.279(2.050, 4.991) 2.539(1.823, 3.698)	4.801(4.844) 6.103(9.753)	
NC *vs* **Concomitant disease**[§] Absent Present	3.830(4.960, 6.940) 3.520 (5.540, 7.500)	6.861(6.791) 6.472(4.022)	0.698
WBC *vs* **Concomitant disease**[§] Absent Present	7.240(5.630, 10.950) 8.140(6.020, 10.560)	9.422(7.095) 9.332(5.058)	0.187
LC *vs* **Concomitant disease**[§] Absent Present	1.040(1.600, 2.470) 1.240(1.690, 2.620)	1.856(1.002) 1.982(1.327)	0.840
NLR *vs* **Margins**[§] Clean Dirty	2.700(1.710, 4.702) 3.405(2.539, 6.839)	4.676(5.367) 7.194(10.300)	0.1171
NC *vs* **Margins**[§] Clean Dirty	3.520 (4.960, 6.500) 4.230(6.900, 10.400)	6.435(6.360) 7.919(4.713)	0.108
WBC *vs* **Margins**[§] Clean Dirty	5.580(7.400, 9.500) 6.020(10.560, 11.820)	9.104(6.684) 10.51(5.852)	0.2146
LC *vs* **Margins**[§] Clean Dirty	1.080 (1.710, 2.540) 0.980 (1.240, 2.470)	1.898(0.971) 1.881(1.549)	0.3815

572 [§]Wilcoxon Rank sum test: [¶]Kruskall-Wallis rank sum test; * statistically significative at 5% level.

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574

575 **Table 3.** Association between Neutrophil-to-Lymphocyte Ratio (NLR), Neutrophil Count (NC),
576 White Blood Cells (WBC), Lymphocyte Count (LC) and size, glycemia and age.
577

578

Variable	†**r**	**95% CI**	‡**t (d.f.)**	***P***
NLR *vs* **Size**	0.315	0.104 – 0.499	2.955(79)	0.004*
NC *vs* **Size**	0.090	0.131 - 0.302	0.805 (79)	0.423
WBC *vs* **Size**	0.054	-0.167 - 0.268	0.478 (79)	0.631
LC *vs* **Size**	- 0.176	-0.380 - 0.044	-1.592 (79)	0.115
NLR *vs* **Glycemia**	0.036	-0.818 - 0.256	0.315(76)	0.734
NC *vs* **Glycemia**	0.070	-0.154 - 0.288	0.619(76)	0.537
WBC *vs* **Glycemia**	0.074	-0150 - 0.292	0.653(76)	0.515
LC *vs* **Glycemia**	0.042	-0.182 - 0.262	0.369 (76)	0.718
NLR *vs* **Age**	0.072	-0.1472 - 0.285	0.646 (80)	0.520
NC *vs* **Age**	0.047	0.1716 - 0.261	0.421 (80)	0.674
WBC *vs* **Age**	0.091	-0.128 - 0.301	0.818 (80)	0.418
LC *vs* **Age**	0.59	-0.159- 0.273	0.534 (80)	0.593

597

598

599 †Pearson's correlation coefficient; ‡t statistics (d.f.) degree of freedom; * statistically significant at
600 5% level
601

602 **Table 4.** Univariate analysis of Neutrophil-to-Lymphocyte Ratio (NLR), Neutrophil Count (NC),
603 White Blood Cells (WBC), Lymphocyte Count (LC) on Local Recurrence and Overall Survival.
604 Cox model results and area under ROC curve.

Variable	**Local recurrence**		**Overall survival**	
	HR (95% C.I.)	***P***	**HR (95% C.I.)**	***P***
Sex M vs F	1.037 (0.449-2.395)	0.93	0.936 (0.558 -.570)	0.802
Age For each 1 year increase	1.046 (0.888-1.232)	0.59	1.15(1.034 -1.278)	0.01*
NLR For each 0.5 increase	1.066 (1.012- 1.122)	0.015*	1.045 (1.005-1.086)	0.028*
LC For each 100 cells increase	1.165 (0.776- 1.75)	0.462	1.103 (0.84-1.448)	0.482
NC For each 1000 cells increase	1.077 (1.025-1.132)	0.003*	1.048 (1.01-1.088)	0.014*
WBCC For each 1000 cells increase	1.078 (1.027 -1.132)	0.002*	1.048 (1.011-1.087)	0.011*
Glycemia For each unitary increase	1.002 (0.994-1.01)	0.631	0.999 (0.993-1.004)	0.68
Concomitant diseases Yes vs no	2.459 (1.02-5.929)	0.045*	1.637 (0.904-2.967)	0.104
Ulceration Yes vs no	5.062 (1.384-18.51)	0.014*	5.29 (2.233-12.54)	< 0.001*
Tumour Size For each cm increase	1.073 (0.954-1.208)	0.241	1.095 (1.025-1.171)	0.007*
Expansive/infiltrative Expansive vs infiltrative	0.267 (0.096-0.746)	0.012*	0.605 (0.351-1.043)	0.071
Necrosis 1 vs 0 2 vs 0 3 vs 0	 1.003 (0.167-6.012) 1.051 (0.263-4.209) 1.844 (0.497-6.841)	0.643 0.998 0.944 0.360	 0.853 (0.290-2.508) 1.173 (0.514-2.678) 1.522 0.675 3.433	0.541 0.772 0.705 0.311
Margins Dirty vs clean	2.584 (1.00- 6.674)	0.050*	2.531 (1.387-4.617)	0.002*
Histotype Other vs Fibrosarcoma	0.893(0.364-2.191)	0.805	1.174 (0.688-2.004)	0.557
Adjuvant.Therapies Yes vs no	1.629 (0.55-4.827)	0.378	1.393 (0.679-2.856)	0.366

605 *Statistically significant at 5% level.

606 **Table 5**. Multivariate analysis of Neutrophil-to-Lymphocyte Ratio (NLR), Neutrophil Count (NC),
607 White Blood Cells (WBC), Lymphocyte Count (LC) on Local recurrence.
608 Results of the Backward selection procedure applied to Cox model to adjust hematological
609 parameters for the variables that were statistically significant in univariate analysis.

Model for NLR*		
Variable	**HR (95% C.I.)**	***P***
NLR For each 0.5 increase	1.064 (1.012 -1.118)	0.016*
Concomitant diseases Yes vs no	2.940 (1.168 -7.404)	0.022*
Ulceration Yes vs no	7.393 (1.835-29.786)	0.005*
Margins Dirty vs clean	2.659 (1.000 -7.072)	0.050*
Model for WBCC**		
Variable	**HR (95% C.I.)**	***P***
WBCC For each 1000 cells increase	1.085 (1.029 -1.144)	0.003*
Concomitant disease Yes vs no	3.647 (1.408 -9.444)	0.008*
Ulceration Yes vs no	5.046 (1.159-21.960)	0.031*
Margins Dirty vs clean	3.055 (1.152 -8.104)	0.025*
Model for NC**		
Variable	**HR (95% C.I.)**	***P***
Neu For each 1000 cells increase	1.084 (1.026 -1.144)	0.004*
Concomitant diseases Yes vs no	3.587 (1.387 -9.277)	0.008*
Ulceration Yes vs no	5.032 (1.143-22.145)	0.033*
Margins Dirty vs clean	3.019 (1.138 -8.007)	0.026*

610 *Statistically significant at 5% level. **Expansile/Infiltrative was removed by the Backward
611 selection because not statistically significant.

612

Table 6. Multivariate analysis of Neutrophil-to-Lymphocyte Ratio (NLR), Neutrophil Count (NC), White Blood Cells (WBC), Lymphocyte Count (LC) on Overall Survival.

Model for NLR**		
Variable	**HR (95% C.I.)**	***P***
NLR For each 0.5 increase	1.028 (0.985-1.073)	0.206
Age For each 1 year increase	1.170 (1.051-1.303)	0.004*
Ulceration Yes vs no	7.352 (2.950-18.325)	<0.0001*
Margins Dirty vs clean	2.337 (1.256 -4.351)	0.007*
Tumour size For each 1 cm increase	1.101 (1.022 -1.186)	0.012
Model for WBCC**		
Variable	**HR (95% C.I.)**	***P***
WBCC For each 1000 cells increase	1.033 (0.993-1.075)	0.111
Age For each 1 year increase	1.171 (1.052 -1.303)	0.004*
Ulceration Yes vs no	6.521 (2.559-16.621)	<0.0001*
Margins Dirty vs clean	2.434 (1.305 -4.541)	0.005*
Tumour size For each 1 cm increase	1.115 (1.037-1.198)	0.003*
Model for NC**		
Variable	**HR (95% C.I.)**	***P***
Neu For each 1000 cells increase	1.032 (0.990-1.076)	0.141
Age For each 1 year increase	1.173 (1.054 -1.306)	0.003*
Ulceration Yes vs no	6.650 (2.614-16.917)	<0.0001*
Margins Dirty vs clean	2.395 (1.286-4.459)	0.006*
Tumor size For each 1 cm increase	1.114 (1.036-1.197)	0.003*

*Statistically significant at 5% level. **NLR, WBCC, NC were removed by Backward selection procedure because not statistically significant. The variable that made NLR, WBCC and NC not significant was ulceration.

Figure legends

Figure 1

Kaplan-Meier estimated local recurrence-free survival probability (continuous line) and 95% confidence intervals (dotted lines).

Figure 2

Kaplan-Meier estimated survival probability (continuous line) and 95% confidence intervals (dotted lines).