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RESEARCH ARTICLE

Application of the Neutrophil-to-Lymphocyte Ratio for Predicting Severity of Hyperthyroidism in Cats

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ABSTRACT

Feline hyperthyroidism progresses with the growth of autonomous thyroid nodules, making the assessment of disease severity necessary for determining appropriate treatment strategies. The neutrophil-to-lymphocyte ratio (NLR), a simple and cost-effective inflammatory biomarker known to be evaluated in inflammatory conditions, systemic illnesses, and malignancies, shows potential as a screening tool for severe hyperthyroidism. In this study, 70 client-owned cats with hyperthyroidism referred for radioiodine therapy were evaluated. Cats were grouped according to thyroid volume as: Group-1; small (< 2cm³), Group-2; medium (2.0–3.9cm³), Group-3; large (4.0–7.9cm³), and Group-4; very large (≥8cm³). Inflammatory biomarkers and total thyroxine levels were measured and compared among groups. Significant differences in NLR were observed among cats of Group-1 and Group-4, with cats in group 4 had a significantly higher NLR than those in group 1 ($P<0.05$). The optimal NLR cut-off for detecting very large (≥8cm³) thyroid tumor was 3.15, with 90% sensitivity, 60.0% specificity and an area under the receiver operating characteristic curve of 0.724, along with a negative predictive value of 97.30%. Significant positive correlation was found between thyroid volume and the NLR ($r=0.283$, $P=0.018$). These findings underscore that an elevated NLR may serve as a reliable predictor of increased tumor volume in cats with hyperthyroidism. In conclusion, the NLR is a valuable inflammatory biomarker for screening severe hyperthyroidism in cats with a high sensitivity and negative predictive value, making it reliable for differentiating severe cases of hyperthyroidism and guiding clinical decisions.

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INTRODUCTION

Feline hyperthyroidism, an animal model of human toxic nodular goiter, is a progressive endocrine disorder (Peterson, 2014). The levels of thyroid hormones increase gradually as the disease progresses due to the growth of autonomous thyroid nodules (Peterson, 2014; Peterson *et al.*, 2016). The management of feline hyperthyroidism can vary depending on severity of the disease. Disease severity may result in antithyroid drug resistance, the need for larger doses of radioiodine therapy, or surgical resection in cases of malignancy (Feldman *et al.*, 2014; Carney *et al.*, 2016). Even cats with presumed benign diseases under medical management may experience sequential dose escalations or drug resistance because adenomatous thyroid tissue may continue to grow (Feldman *et al.*, 2014; Carney *et al.*, 2016). Conventional methods, such as computed tomography, scintigraphy and biopsy, are options for assessing and differentiating thyroid disease severity, including the size of nodules and the possibility of thyroid carcinoma. However, applications of these methods are limited by their invasiveness, high cost, and the necessity of anesthesia. Therefore, the use of inflammatory biomarkers has become an attractive noninvasive diagnostic option for assessing thyroid disease severity.

The neutrophil-to-lymphocyte ratio (NLR), which is simple and cost-effective, is an important indicator that tends to increase in cases of inflammation, serious systemic illnesses or the presence of malignancies (Zahorec, 2001; Zahorec, 2021). The NLR is influenced by opposite tendencies for neutrophil and lymphocyte counts in various conditions such as immune responses and neuroendocrine hormone secretion (Zahorec, 2021). However, to our knowledge, the relationship between disease severity and the NLR in cats with hyperthyroidism has not been investigated in feline medicine. As hyperthyroidism

progresses, the enlargement of autonomous thyroid nodules leads to excessive hormone secretion and affects systemic metabolism, resulting in physiological stress that can increase the NLR. Additionally, thyroid malignancy can be associated with cancer-associated inflammation or carcinogenesis, contributing to an increased NLR.

Thus, it was hypothesized that the NLR increases in proportion to the severity of feline hyperthyroidism and may serve as a noninvasive biomarker to help identify cats with more advanced disease. Therefore, this study was planned to evaluate the predictive value of NLR when screening cats with severe hyperthyroidism, based on previous investigations showing that inflammatory biomarkers are increased in serious systemic diseases and tumors.

MATERIALS AND METHODS

Experimental animals: A retrospective review of 85 hyperthyroid cats referred to the Veterinary Teaching Hospital of Chungbuk National University, Chungbuk, Korea for radioiodine therapy during the period from May 2020 to September 2023, was conducted with ethics approval (CBNUA-2007-22-01) and owner's consent. Cats were diagnosed with hyperthyroidism and included in the study based on serum thyroxine (T4) concentration >4.7µg/dL and increased technetium-99m uptake of the thyroid gland on scintigraphy. Fifteen cats were excluded because they did not meet the inclusion criteria, had no blood analysis results for key parameters, including complete blood count (CBC), serum amyloid A (SAA), and albumin or had concurrent diseases. Administration of antithyroid drugs to selected cats was stopped for 7 to 14 days before the examination.

Thus, a total of 70 cats were included in this study, consisting of 33 mixed breeds (domestic short hair) and 37 pure breeds (12 Russian blues, nine Persians, five American short hairs, three Norwegian forest cats, three Siamese, one Neva masquerade, one Bengal, one British short hair, one Turkish angora, and one Scottish fold). The cats aged between 6 and 17 years (median 12 and IQR 10–13.25); 29 (41.43%) cats were neutered males and 41 (58.57%) were spayed females. The median body weight of the cats was 4.25 (IQR 3.76–5.32), and no concurrent diseases were identified at the time of assessment. The duration of the disease, indicating the time interval between diagnosis and scintigraphy, ranged from 0 to 48 months (median 3 months and IQR 2–7.25).

Blood analysis: Blood samples (approximately 3–5mL) were collected from the cephalic or saphenous vein. For the CBC, blood was drawn into tubes containing EDTA as an anticoagulant. Serum samples were collected in serum separation or plain tubes and were allowed to clot at room temperature for 30 minutes before being centrifuged at 1500×g for 10 minutes to separate the serum. Serum samples analyzed in-house were tested immediately after centrifugation. For external analyses, serum samples were sent to the referral laboratory on the same day under refrigerated conditions (2–8°C) without freezing. The CBC was performed using a hematology analyzer (IDEXX ProCyte Dx, IDEXX Laboratories, Westbrook, ME, USA) at 20°C. Serum albumin was measured using a biochemical analyzer (Hitachi 7020, Hitachi High-Technologies Co., Tokyo, Japan), and serum amyloid A (SAA) levels were determined by immunofluorescence quantification analyzer (VET chroma, ANIVET Inc., Chuncheon, South Korea).

The neutrophil-to-lymphocyte, platelet-to-lymphocyte and monocyte-to-lymphocyte ratios were calculated by dividing the absolute numbers (cells/µL) of neutrophil, platelet, and monocyte by that of lymphocyte. The neutrophil-to-platelet ratio was calculated by dividing the absolute neutrophil count by the absolute platelet count. Neutrophil percentage-to-albumin and SAA-to-albumin ratios were calculated by dividing the neutrophil percentage and SAA concentration by serum albumin concentration, respectively.

Serum T4 concentration was measured using chemiluminescent enzyme immunoassay analyzer (Immulite 2000, Siemens Healthcare Diagnostics, Deerfield, IL, USA) as an in-house assay, using a kit for total T4 (Catalog No. L2KCT2, Siemens Healthcare Diagnostics, Llanberis, UK). The analytical sensitivity of the assay was 0.12µg/dL. According to manufacturer data, the intra-assay coefficient of variation ranged from 3.7 to 5.9%, and the inter-assay coefficient of variation ranged from 6.7 to 12.7%, depending on the concentration level. If the concentration exceeded the upper detection limit of the in-house system (>15µg/dL), samples were sent to a commercial laboratory (IDEXX laboratory, Inc, Westbrook, ME, USA) for further analysis. If the measurement exceeded the upper limit (>24µg/dL) at the commercial laboratory and was not measurable, it was assumed as 25µg/dL.

Thyroid scintigraphy: Thyroid scintigraphy was performed using a gamma camera (Dilon 6800 Gamma Camera; Dilon Technologies, Newport News, VA) with a low-energy, high-resolution parallel-hole collimator. Static thyroid images were obtained for a total of 200,000 counts after 40–60 minutes following intravenous administration of 3–4mCi of technetium-99m (New Korea Industrial, Seoul, Korea), as described by Peterson and Broome (2015).

The elimination of inter-operator variability was based on a single observer's measurement of all thyroid volumes. Thyroid volume was estimated using the equation for a prolate spheroid, $V=4/3\ \pi a^2c$, where 'a' represents the semi-minor axis and 'c' represents the semi-major axis of each thyroid nodule, while 'V' stands for thyroid volume. The total thyroid volume was calculated by summing the volume estimations of all identified thyroid nodules (Peterson *et al.*, 2016). For the assessment of disease severity, the thyroid-to-salivary ratio (TSR) and thyroid-to-background ratio (TBR) were calculated by dividing the mean thyroid radioactivity by that of the salivary gland and background region, respectively. The salivary gland radioactivity was measured by manually drawing a region of interest over the visible zygomatic/molar salivary gland on the static scintigraphy images (Peterson and Broome, 2015). Cats were grouped by tumor size into small (<2.0cm^3), medium (2.0–3.9cm^3), large (4.0–7.9cm^3), or very large (≥8cm^3), as described earlier (Peterson *et al.*, 2016).

Statistical analyses: Statistical analyses were performed using SPSS (version 27.0; IBM Corp, Armonk, NY, USA) and GraphPad Prism (version 10.0.0; GraphPad Software, Boston, MA, USA). The Kolmogorov–Smirnov test was used to determine normal distribution. Medians (interquartile ranges [IQRs]) represented non-normally distributed data, whereas means with 95% confidence intervals [Cis]) represented normally distributed data. The Kruskal-Wallis test and Dunn's post hoc test were used to assess associations between variables. Receiver operating characteristic (ROC) curve analysis was applied to determine the discriminatory power of the total T4, NLR, and SAA for detecting very large thyroid tumors, with area under the curve (AUC) and optimized cut-offs for sensitivity and specificity. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using 2×2 tables in Microsoft 2019 Excel software (Microsoft Corp, Redmond, WA, USA). Correlations between thyroid volume, total T4, and inflammatory biomarkers were examined, with partial correlations controlling for confounders such as thyroid volume and total T4.

RESULTS

Scintigraphic characteristics: Among the study population of 70 cats, 44 (62.86%) exhibited a single thyroid nodule, 25 (35.71%) had two thyroid nodules, and one (1.43%) presented with multifocal thyroid nodules. The mean value and the standard deviation of the thyroid-to-salivary ratio (TSR) was 7.80±6.60 (95% CI, 6.22–9.37) and that of the thyroid-to-background ratio (TBR) was 24.33±19.45 (95% CI, 19.69–28.97). The mean volume of the thyroid tumor was 4.97±3.56cm^3, with a 95% CI ranging from 4.12 to 5.82cm^3.

Comparisons among the thyroid volume groups: The study population was divided into four groups based on thyroid volume: The groups were small, medium, large and very large. Scintigraphic images were displayed using a rainbow color scale, in which color intensity corresponded to the counts per pixel, reflecting the degree of radiotracer uptake in the thyroid (Fig. 1). Small tumors were recorded in 10 cats (14.3%), medium in 25 (35.7%), large in 25 (35.7%), and very large in 10 cats (14.3%). Scintigraphic images for small, medium, large and very large tumors are

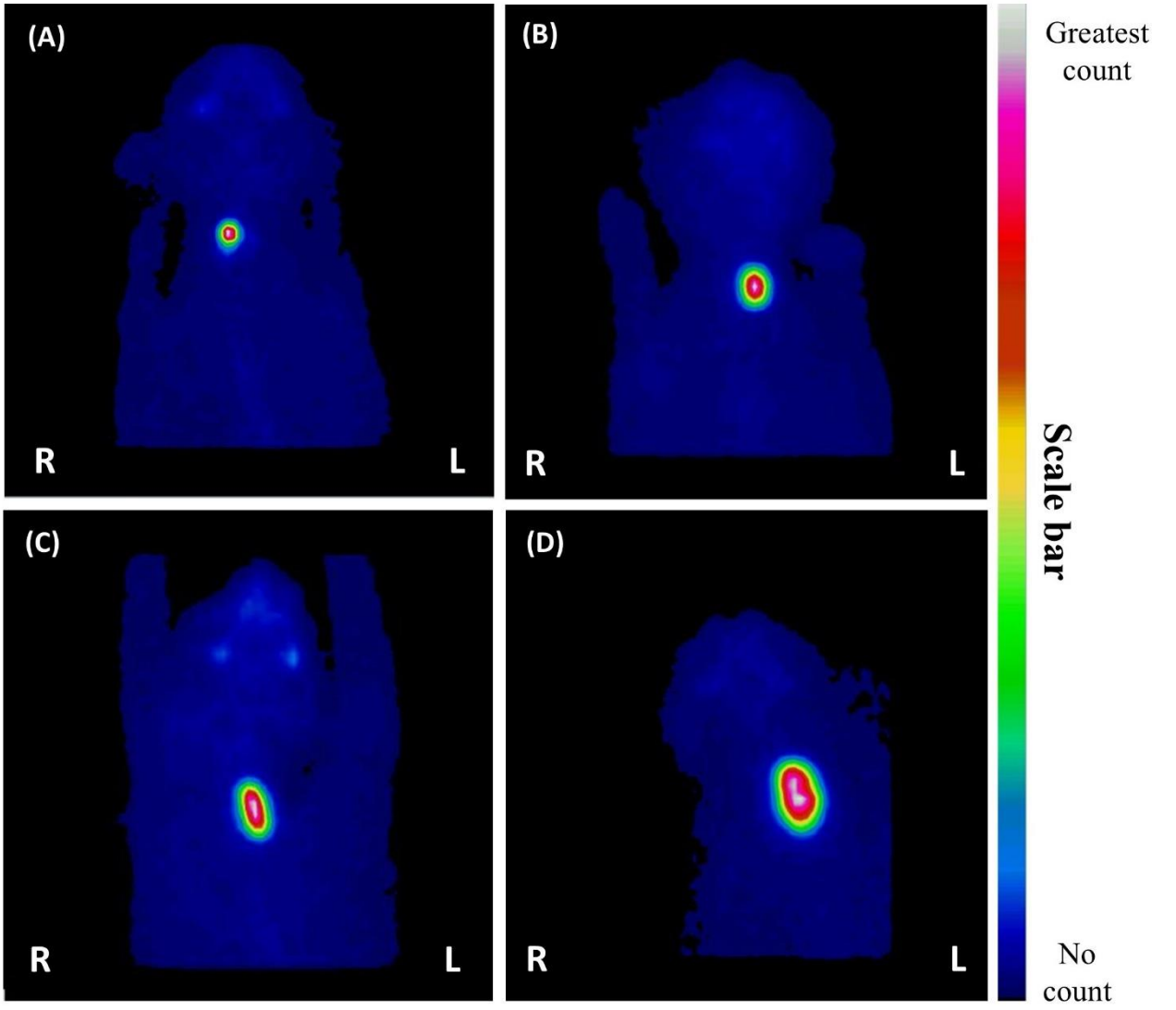

Fig. 1: Scintigraphic images for the thyroid in each thyroid volume group. The color display pattern of the rainbow is used in this planar scintigraphy image. The color shade represents the counts per pixel. The tumor volumes for each image are as follows: (A) small group, 1.15cm^3; (B) medium group, 3.73cm^3; (C) large group, 4.58cm^3; and (D) very large group, 9.88cm^3.

shown in Fig. 1A to Fig. 1D. Several factors related to disease severity (Fig. 2) and inflammatory biomarkers were compared between the groups (Table 1), as described below:

Disease severity: Disease duration was significantly different among the four volume groups (*P*=0.034), with a gradual increasing trend was observed from group 1 to group 3, although non-significant differences were found in post hoc pairwise comparisons (Fig. 2A). Thyroid volume was significantly different between the volume groups (*P*<0.001). Group 4 (median 10.52 [IQR 8.75–16.50]) had a higher thyroid volume than groups 1 (median 1.60 [IQR 1.17–1.76]; P<0.001) and 2 (median 3.23 [IQR 2.52–3.61], P<0.001). Group 3 (median 5.34 [IQR 4.43–6.31]) also had a significantly higher thyroid volume than group 1 (P<0.001) and group 2 (P<0.001; Fig. 2B).

The TSR, TBR and total T4 levels were significantly different among the four volume groups (P<0.001), with significantly higher values in groups 3 and 4 compared to groups 1 and 2 (P<0.05). The TSR increased from a median of 3.34 [IQR 2.34–6.01] in group 1, 4.11 [3.02–6.88] in group 2 and 7.77 [4.12–12.07] in group 3, to 10.52 [5.64–20.43] in group 4. The TBR rose from 10.52 [7.14–16.95] in group 1, 14.71 [8.32–21.20] in group 2 and 23.32 [13.54–41.30] in group 3, to 37.47 [21.48–43.40] in group 4. The total T4 levels were also elevated across groups: 8.10 [6.34–10.10] in group 1, 12.50 [9.64–13.30] in group 2, 14.60 [12.85–16.05] in group 3, and 15.30 [14.30–19.53] in group 4. Detailed statistical comparisons are presented in Figs. 2C–2E.

Inflammatory biomarkers: Significant variations in neutrophil counts were observed between the volume groups (P=0.006). Specifically, group 4 (median 8.67 [IQR 6.27–12.43]) demonstrated a significantly higher count than group 1 (median 3.49 [IQR 2.12–5.17], P<0.01), while non-significant differences were observed among the other groups. There were significant differences in the NLR among the volume groups (P=0.032), where group 4 (median 3.82 [IQR 3.35–5.96]) exhibited a significantly higher NLR (P<0.05) than group 1 (median 1.58 [IQR 1.02–4.20]). Platelet count also showed a borderline significant difference among the groups (P=0.050), although non-significant differences were found in the post hoc analysis. In contrast, the remaining inflammatory biomarkers, including the lymphocyte count, neutrophil-to-platelet, platelet-to-lymphocyte, neutrophil percentage-to-albumin, monocyte-to-lymphocyte, and SAA-to-albumin ratios; and SAA and albumin levels, were not significantly different among the volume groups (Table 1).

ROC curve analysis and optimal cut-offs of the NLR and SAA and total T4 levels: The ROC curve was used to determine the diagnostic roles of the NLR and total T4 and SAA levels in distinguishing very large thyroid tumors (Fig. 3). The optimal cut-off (sensitivity and specificity)

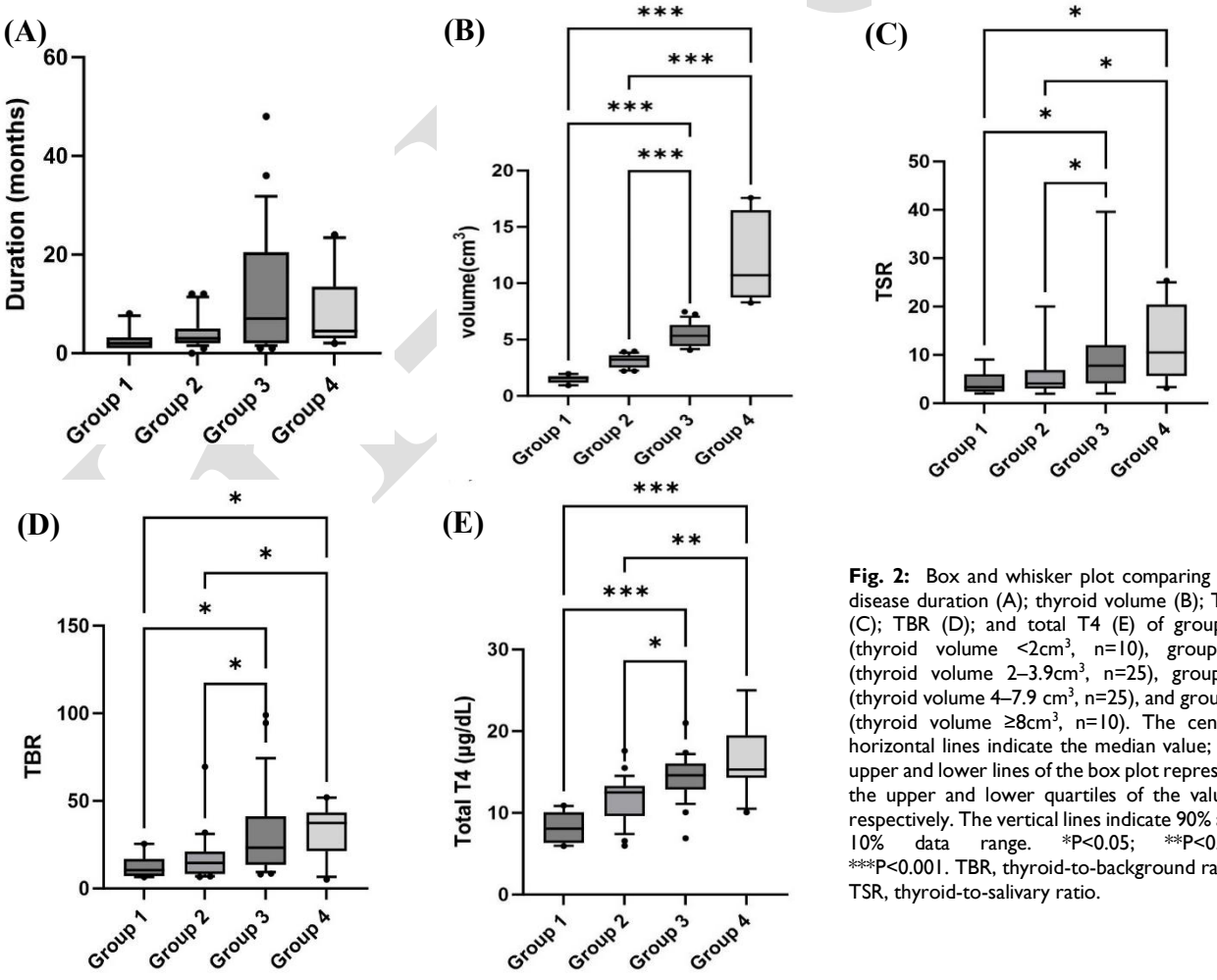

Fig. 2: Box and whisker plot comparing the disease duration (A); thyroid volume (B); TSR (C); TBR (D); and total T4 (E) of group 1 (thyroid volume <2cm^3, n=10), group 2 (thyroid volume 2–3.9cm^3, n=25), group 3 (thyroid volume 4–7.9 cm^3, n=25), and group 4 (thyroid volume ≥8cm^3, n=10). The central horizontal lines indicate the median value; the upper and lower lines of the box plot represent the upper and lower quartiles of the values, respectively. The vertical lines indicate 90% and 10% data range. *P<0.05; **P<0.01; ***P<0.001. TBR, thyroid-to-background ratio; TSR, thyroid-to-salivary ratio.

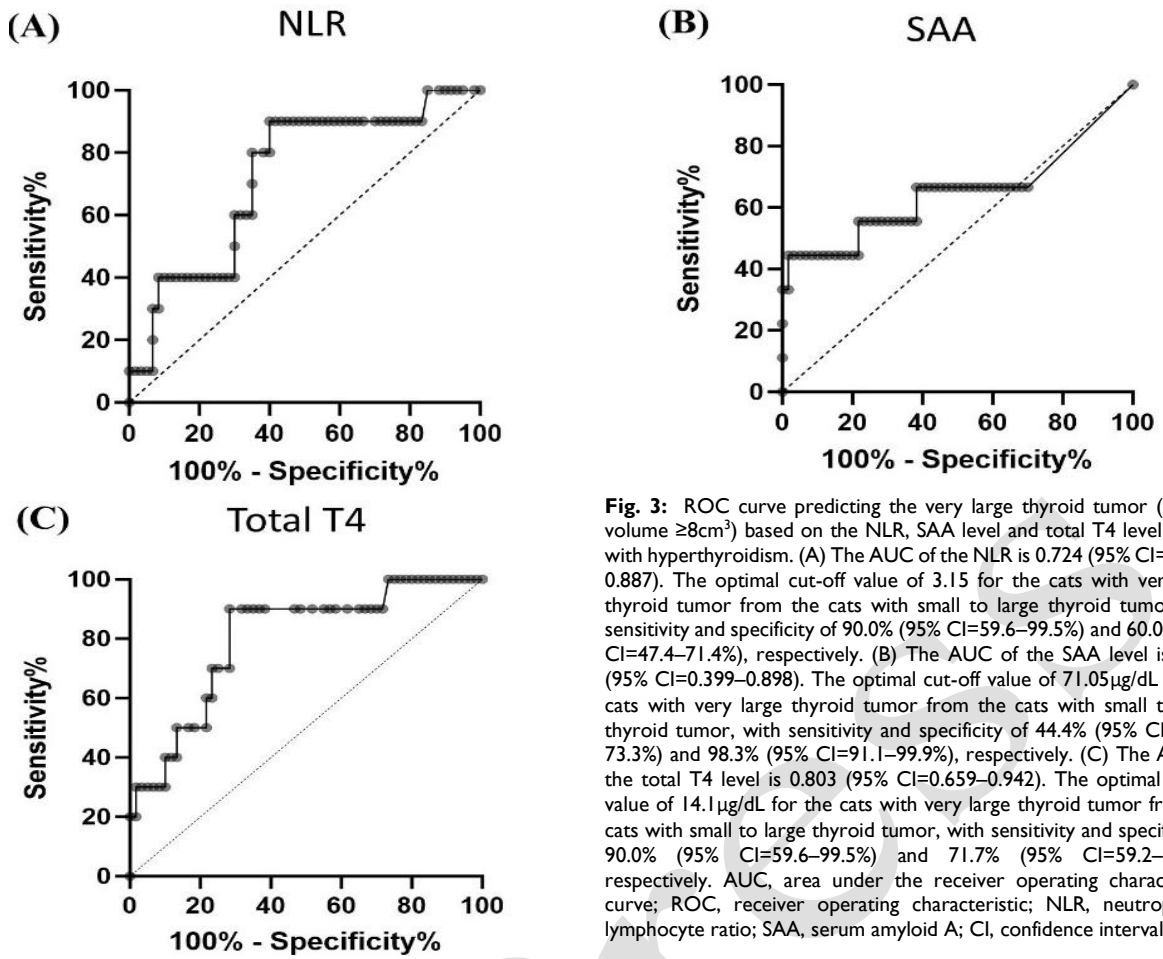

Fig. 3: ROC curve predicting the very large thyroid tumor (thyroid volume ≥8cm^3) based on the NLR, SAA level and total T4 level in cats with hyperthyroidism. (A) The AUC of the NLR is 0.724 (95% CI=0.561–0.887). The optimal cut-off value of 3.15 for the cats with very large thyroid tumor from the cats with small to large thyroid tumor, with sensitivity and specificity of 90.0% (95% CI=59.6–99.5%) and 60.0% (95% CI=47.4–71.4%), respectively. (B) The AUC of the SAA level is 0.648 (95% CI=0.399–0.898). The optimal cut-off value of 71.05μg/dL for the cats with very large thyroid tumor from the cats with small to large thyroid tumor, with sensitivity and specificity of 44.4% (95% CI=18.9–73.3%) and 98.3% (95% CI=91.1–99.9%), respectively. (C) The AUC of the total T4 level is 0.803 (95% CI=0.659–0.942). The optimal cut-off value of 14.1μg/dL for the cats with very large thyroid tumor from the cats with small to large thyroid tumor, with sensitivity and specificity of 90.0% (95% CI=59.6–99.5%) and 71.7% (95% CI=59.2–81.5%), respectively. AUC, area under the receiver operating characteristic curve; ROC, receiver operating characteristic; NLR, neutrophil-to-lymphocyte ratio; SAA, serum amyloid A; CI, confidence interval.

Table 1: Comparison of selected inflammatory and hematologic parameters across thyroid-volume groups in cats with hyperthyroidism

Blood Parameters	Group1(vol.<2cm^3; 10 cats)	Group2 (vol.2-3.9cm^3; 25 cats)	Group3 (vol.4-7.9cm^3; 25 cats)	Group4 (vol.>8cm^3; 10 cats)	P-value
Neutrophil (×10^3/μL)	3.49 (2.12-5.17)	5.37 (3.92-6.75)	5.60 (3.63-8.27)	8.67[a] (6.27-12.43)	0.006**
Lymphocyte (×10^3/μL)	2.04 (1.40-2.73)	2.31 (1.58-3.67)	1.66 (1.34-2.11)	2.10 (1.40-2.83)	0.18
Platelet (×10^3/μL)	264.5 (195.0-318.5)	326.0 (251.0-414.0)	284.0 (216.5-353.0)	427.0 (269.0-510.5)	0.05
NLR	1.58 (1.02-4.20)	2.44 (1.58-3.38)	3.51 (1.81-4.95)	3.82[a,b] (3.35-5.96)	0.032*
NPR	0.015 (0.010-0.020)	0.020 (0.010-0.030)	0.020 (0.010-0.030)	0.025 (0.018-0.050)	0.21
PLR	127.93 (96.41-176.54)	121.85 (97.77-188.17)	143.39 (110.65-235.49)	167.14 (119.66-289.62)	0.37
NP/Alb	22.66 (18.00-29.61)	29.19 (24.22-32.05)	28.77 (19.58-34.65)	25.55 (22.50-30.69)	0.48
MLR	0.09 (0.06-0.23)	0.13 (0.09-0.20)	0.17 (0.13-0.26)	0.23 (0.13-0.30)	0.15
SAA/Alb	2.23 (2.00-8.67)	3.05 (2.17-5.27)	6.44 (2.80-12.89)	13.43 (1.72-44.10)	0.25
SAA (mg/dL)	4.90 (4.90-23.27)	7.02 (4.90-12.36)	15.65 (7.93-35.36)	26.85 (4.90-102.1)	0.066
Alb (g/dL)	2.45 (2.28-2.60)	2.30 (2.10-2.45)	2.30 (2.15-2.85)	2.60 (2.20-3.45)	0.19

Data shown as medians and interquartile ranges. Kruskal–Wallis test. *P<0.05 among groups; **P<0.01 among groups. a. P<0.05, compared with Group 1, post hoc Dunn's multiple comparison test. b. P<0.05, compared with Group 2, post hoc Dunn's multiple comparison test. Alb, albumin; MLR, monocyte-to-lymphocyte ratio; NLR, neutrophil-to-lymphocyte ratio; NP/Alb, neutrophil percent to albumin ratio; NPR, neutrophil-to-platelet ratio; SAA, serum amyloid A; SAA/Alb, serum amyloid a to albumin ratio; vol., volume.

values were as follows: 1) NLR>3.15 (90.0% and 60.0%, P=0.024; Fig. 3A); 2) SAA>71.05mg/dL (44.4% and 98.3%, P=0.15; Fig. 3B); and 3) total T4>14.1μg/dL (90.0% and 71.7%, P=0.003; Fig. 3C).

The PPV and NPV were evaluated to identify severe hyperthyroidism. The optimal cut-off value of 3.15 for the NLR exhibited a high NPV of 97.30% and a PPV of 27.27%. For the SAA level, with an optimal cut-off value of 71.0mg/dL, the PPV and NPV were 80.00 and 92.19%, respectively. The total T4 level, with an optimal cut-off value of 14.1μg/dL, revealed a high NPV of 97.73% and a PPV of 32.14% (Table 2).

Correlation of inflammatory biomarkers and total T4 level with thyroid volume: Thyroid volume was significantly positively correlated with the total T4 (r=0.499, P<0.001; Fig. 4A) and SAA levels (r=0.403, P=0.001; Fig. 4B), neutrophil count (r=0.322, P=0.007, Fig. 4C), and NLR (r=0.283, P=0.018; Fig. 4D). However, lymphocyte counts (r=-0.056, P=0.65, Fig. 4E) were not significantly correlated with thyroid volume.

Table 2: Predictive values of the NLR, SAA level, and total T4 levels for very large goiter in cats with hyperthyroidism

Variables	Threshold	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
NLR	3.15	90.00	60.00	27.27	97.30
	3.39	80.00	65.00	27.59	95.12
	5.66	40.00	91.67	44.44	90.16
SAA	25.7	55.56	78.33	27.78	92.16
(mg/dL)	71.0	44.44	98.33	80.00	92.19
total T4	14.1	90.00	69.35	32.14	97.73
(μg/dL)	15.6	50.00	85.48	35.71	91.38
	17.7	27.27	98.39	75.00	88.41

NLR, neutrophil-to-lymphocyte ratio; NPV, negative predictive value; SAA, serum amyloid A; PPV, positive predictive value; T4, thyroxine.

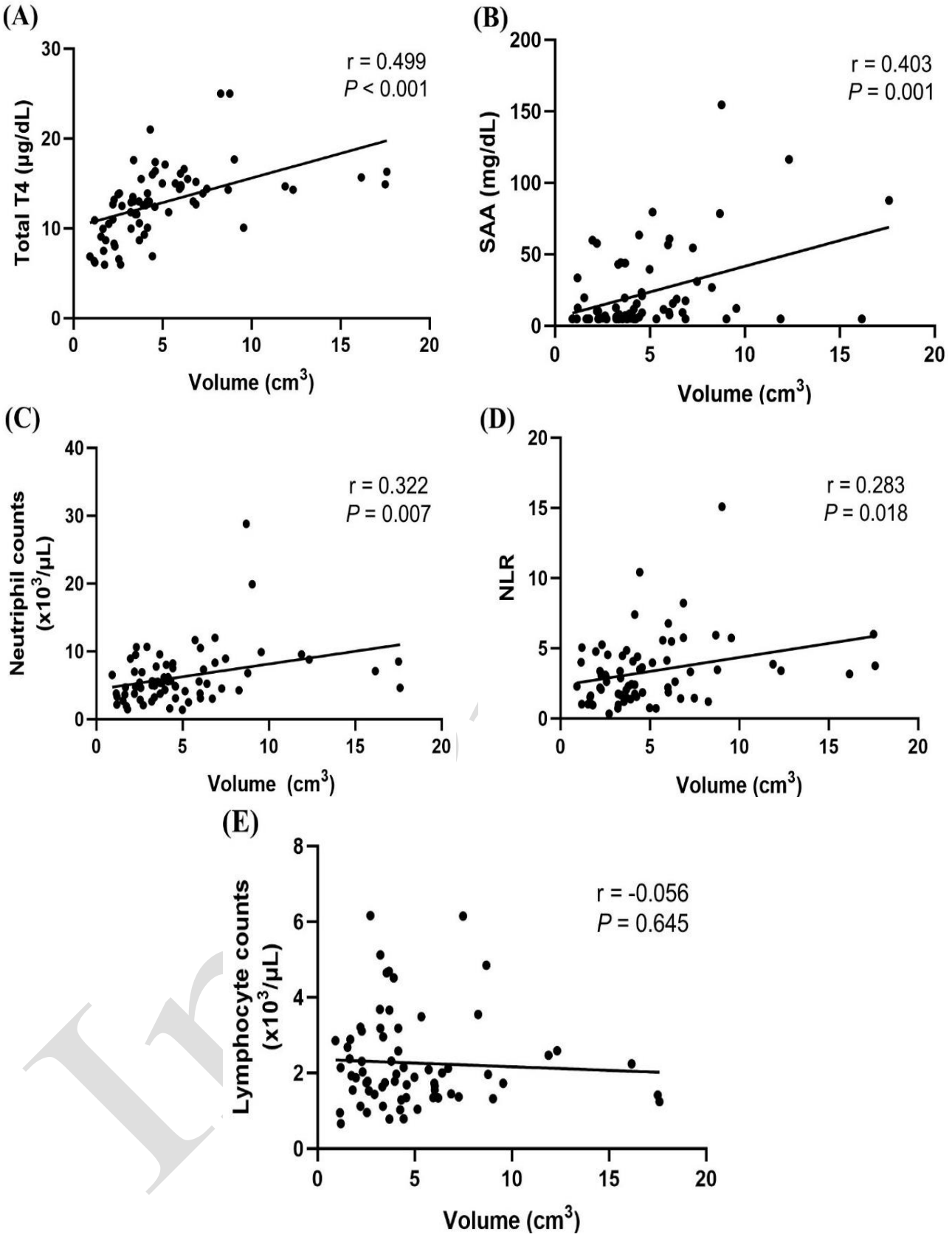

Fig. 4: Correlations between (A) thyroid volume and total T4 level; (B) thyroid volume and SAA, (C) thyroid volume and neutrophil counts, (D) thyroid volume and the NLR, and (E) thyroid volume and lymphocyte counts in cats with hyperthyroidism. SAA, serum amyloid A; NLR, neutrophil-lymphocyte ratio.

The total T4 level was significantly correlated with the SAA level (r=0.411, P<0.001; Fig. 5A), but not with the neutrophil count (r=0.153, P=0.21, Fig. 5B), NLR (r=0.189, P=0.12; Fig. 5C) or lymphocyte count (r=-0.040, *P*=0.74, Fig. 5D). The SAA level maintained a significantly positive correlation with thyroid volume after adjusting for the total T4 level (P=0.047) and with the total T4 level after adjusting for thyroid volume (P=0.034).

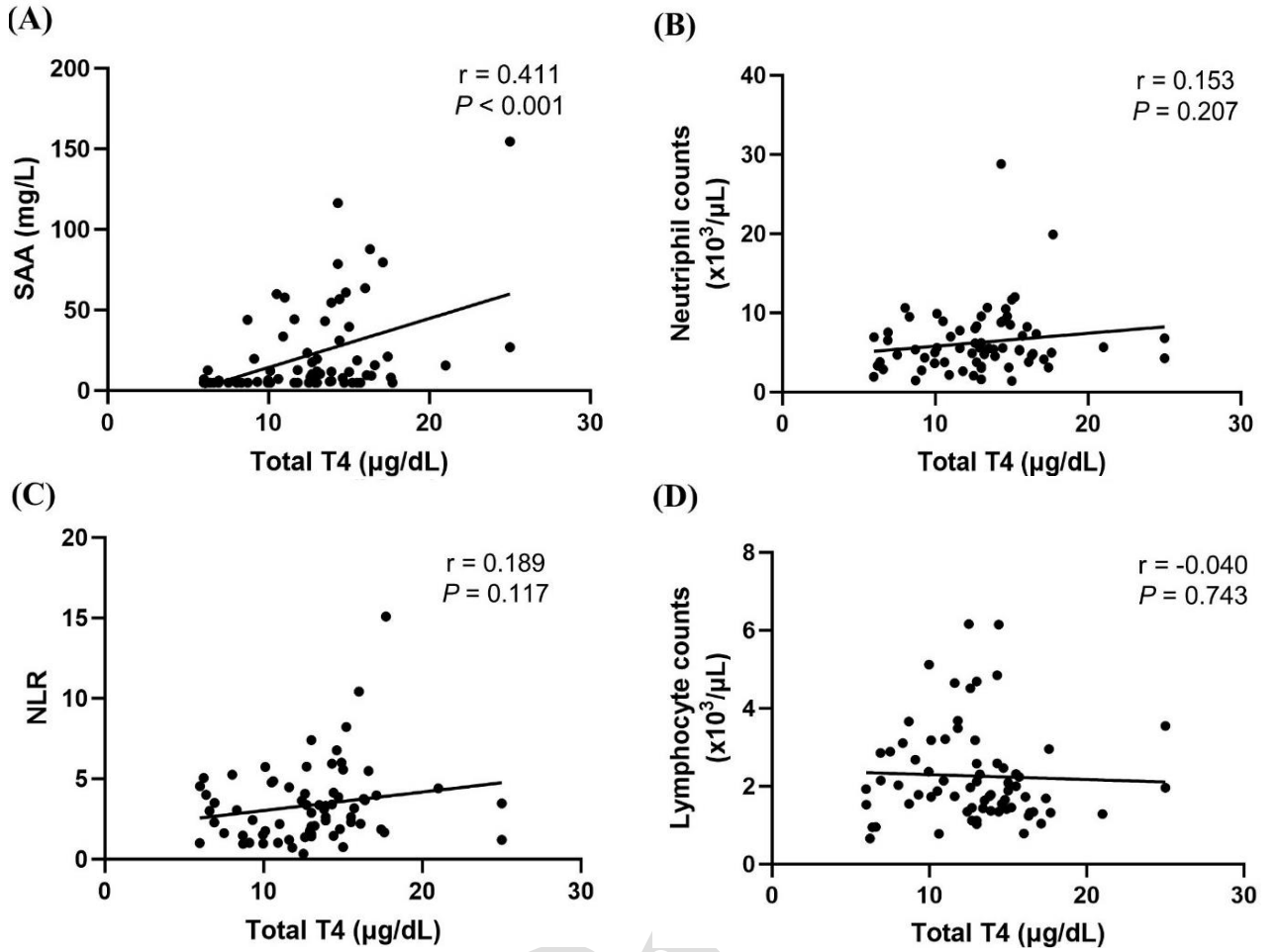

Fig. 5: Correlations between: (A) Total T4 level and SAA levels; (B) Total T4 level and neutrophil counts; (C) the total T4 level and NLR, and (D) the total T4 level and lymphocyte counts in cats with hyperthyroidism. SAA, serum amyloid A; NLR, neutrophil-to-lymphocyte ratio.

DISCUSSION

In this study, the NLR of cats with hyperthyroidism varied among different thyroid volume groups, with group 4 showing significantly higher values compared to groups 1 and 2. Notably, cats with the largest thyroid volume exhibited the highest NLR. This tendency of an increased NLR with a greater thyroid volume in cats with hyperthyroidism aligns with the notion that inflammation is associated with disease severity. These findings are parallel to observations in human medicine, where an elevated NLR was linked to various health conditions and was correlated with disease severity (Zahorec, 2021). However, it is important to acknowledge that specific NLR reference ranges in cats are essential for precise clinical interpretation and warrant further investigations.

The NLR previously reported in healthy cats ranged from 1 to 2, corresponding to the results found in healthy humans (Gori *et al.*, 2021; Bojarski *et al.*, 2022; Fries *et al.*, 2022; Tuna and Kirkulak, 2023). An elevated NLR was identified in cats with hyperthyroidism, except in the small tumor-volume group (<2cm³) in this study, which could be owing to a suspicious concurrent inflammatory response or stress reaction in cats with larger thyroid tumors. When the tumor volume was larger than that of group 1 in this study, the NLR values for the median tumor-volume group exhibited similarities to values reported in previous studies for cats with other mild systemic diseases (Bojarski *et al.*, 2022; Fries *et al.*, 2022; Tuna and Kirkulak, 2023). Moreover, the value of the NLR for the large to very large tumor-volume groups corresponded to those reported in cats with severe systemic illnesses, such as heart failure (NLR 5.11) and systemic inflammatory response syndrome or sepsis (NLR 4.53) (Gori *et al.*, 2021; Fries *et al.*, 2022). This suggests that the increased inflammatory response with a larger thyroid tumor volume, which is indicated by the NLR, may be comparable to the increase in disease severity in cats.

Additionally, the increased NLR due to carcinogenesis or inflammatory responses in tumors, was correlated with tumor volume (Zahorec, 2021), and has been reported in cats with malignant tumors, such as feline malignant mammary tumors and injection site sarcomas (Chiti *et al.*, 2020; Naito *et al.*, 2021). The present study also identified an increase in the NLR in larger thyroid tumors and a positive correlation with thyroid volume. This finding is in agreement with previous reports in feline malignancies, where NLR was associated with tumor burden and prognosis. In feline malignant mammary tumors, a high NLR was significantly associated with shorter survival (P<0.01) and showed predictive value for one-year survival (cut-off value 5.67) (Naito *et al.*, 2021). Similarly, in cats with feline injection site sarcoma, NLR was significantly correlated with tumor size (P=0.004), and the optimal NLR cut-off value for predicting local recurrence at 2 and 3 years was 3.654 (Chiti *et al.*, 2020). Although the etiology of

NLR elevation may differ between these conditions, the elevation of NLR values highlights the existence of systemic inflammation in feline hyperthyroidism, which may be more pronounced in cases with larger thyroid volumes that may be malignant.

To the best of our knowledge, the NLR has not been evaluated in feline hyperthyroidism but has been reported in human patients with thyroid nodules as a method to distinguish between benign and malignant cases (Zeren *et al.*, 2017). According to these workers, the NLR values were higher in malignant thyroid nodules than in benign ones, similar to the values identified in cats with very large thyroid tumors in this study. Moreover, a previous study on feline hyperthyroidism reported that the very large size of the thyroid could be related to progressive disease and the possibility of malignancy (Peterson *et al.*, 2016). Feline hyperthyroidism could be considered as an animal model for toxic nodular goiter in humans, and both conditions involve the potential progression to thyroid malignancy (Smith *et al.*, 2013; Peterson, 2014; Tam *et al.*, 2019; Mohamed *et al.*, 2022). These similarities in the NLR between severities, including the possibility of malignancy, feline hyperthyroidism, and malignant thyroid tumors in humans, are noteworthy. However, the differentiation of malignancy through histopathology was not included in this study because it was conducted solely to screen for inflammatory biomarkers in cats with hyperthyroidism during the initial diagnosis. Therefore, further studies are needed to evaluate the NLR of malignant thyroid tumors, including its correlation with tumor size, in cats with hyperthyroidism.

When considering the optimal cut-off value for the NLR while screening cats with severe hyperthyroidism, the identified threshold value of 3.15 emerged as a clinically relevant point of consideration. The sensitivity of 90% indicated the high ability of the NLR to identify cats with very large thyroid tumors, suggesting that it as a reliable tool for detecting severe cases. The specificity of 60% implies that the NLR may produce false positives, indicating that a proportion of cats without severe hyperthyroidism may be detected. Nonetheless, the strong NPV of 97.30% indicates that the NLR is excellent at eliminating severe situations, reducing the possibility of overlooking critical conditions. The PPV of 27.27% indicated that while a positive NLR result was not an indicative of severe hyperthyroidism, the high NPV reinforced the utility of the NLR as a valuable screening inflammatory biomarker, particularly in its ability to reliably exclude severe hyperthyroidism. These considerations underscore the importance of the chosen cut-off value of the NLR for screening tests.

Another inflammatory biomarker, the SAA level, which is a major acute-phase protein in cats, has been previously studied in cats with hyperthyroidism; however, it remained complex and has shown conflicting results (Yuki *et al.*, 2020; Glück *et al.*, 2022). In the present study, the relationship between the SAA level and thyroid volume was assessed. Although there were non-significant differences in SAA levels among the thyroid volume groups, there was a significantly positive correlation between SAA levels, thyroid volume, and total T4 levels. These variations indicate the complex nature of feline hyperthyroidism and the possibility of differences in inflammatory responses. The SAA is produced by the liver; however, it is also produced from adipose and various other tissues (den Hartigh *et al.*, 2023). Considering that thyroid hormones play a pivotal role in regulating metabolism, including energy expenditure and fat metabolism, it is plausible that their overproduction can lead to systemic effects, including alterations in SAA levels. The non-specific nature of SAA, which is affected by various metabolic and inflammatory statuses, suggests that the inflammatory response due to severe hyperthyroidism may not be the only factor contributing to its elevation. Therefore, a cautious interpretation of the association between elevated SAA levels and disease severity is required.

Another notable finding was the significantly higher neutrophil counts in group 4 compared to group 1, although non-significant differences were observed among the other groups, which also correlated positively with thyroid volume, but not with the total T4 level. Progressive hyperthyroidism plays a crucial role in inflammation and stress, resulting in neutrophilia and lymphopenia, owing to the fact that the opposite changes in neutrophil and lymphocyte counts are a multifactorial dynamic process depending on fine-tuning and regulation of various immunologic, neuroendocrine, humoral and biologic processes; the influence of stress hormones and sympathetic/parasympathetic imbalance of the vegetative nervous system (Zahorec, 2021).

However, lymphopenia was not observed, and a lack of correlation between the severity of hyperthyroidism and lymphocyte counts was observed in this study. This can be explained by the intricate interplay between thyroid hormone levels and immune responses. Thyroid hormones are known to play a pivotal role in immune modulation (Wenzek *et al.*, 2022). On the surface of T and B lymphocytes, thyroid hormone receptors, such as thyroid hormone receptor alpha and beta, exist, suggesting that thyroid hormones have the potential to directly influence lymphocyte proliferation and activation (Wenzek *et al.*, 2022). It may be crucial for primary B cell development, as evidenced by studies indicating reduced numbers of pro-B cells and impaired pro-B cell proliferation in the absence of thyroid hormone receptor signaling in murine models of thyroid hormone receptor resistance (Arpin *et al.*, 2000; Park *et al.*, 2021).

Moreover, *in-vitro* stimulation of human peripheral blood B lymphocytes with triiodothyronine has been shown to lead to accelerated B cell proliferation, highlighting the direct impact of thyroid hormones on B cell activity (Wenzek *et al.*, 2022). Additionally, in T lymphocytes, *in-vivo* application of T4 has been shown to enhance T cell numbers and promote antitumor immunity in a murine lymphoma model (Wenzek *et al.*, 2022). Therefore, in the context of a state of over-secretion of thyroid hormones, it is necessary to consider the potential effects of thyroid hormones on lymphocyte dynamics. Although lymphocyte counts can decrease in response to stress or inflammation, the systemic effect of thyroid hormones accelerates the proliferation and development of lymphocytes, potentially counteracting the effects of stress or inflammation and masking the expected decrease in the lymphocyte count. This multifaceted relationship between the immune response in cats with hyperthyroidism, which

is an animal model for toxic nodular goiter, and the complex interplay between thyroid hormones and immune cell dynamics needs cautious understanding based on further advanced studies for veterinary medicine.

This study had some limitations. The effect of methimazole on the NLR in cats with hyperthyroidism could not be investigated. Methimazole, a commonly prescribed antithyroid drug for feline hyperthyroidism, has a plasma half-life of 6h, and the duration of its pharmacodynamic effect can last for >20h owing to its ability to accumulate in the thyroid gland (Trepanier *et al.*, 1991). Because this study was aimed to investigate the use of the NLR as a screening tool at the time of diagnosis, the antithyroid drug medication in all cats was stopped for >1 week. However, the systemic condition of cats, which was well controlled until the withdrawal of medication, could be different from that of cats with uncontrolled hyperthyroidism. Therefore, further studies on the NLR in well-controlled antithyroid medication or radioiodine therapy could be valuable in discriminating against the systemic effects of the over-secretion of thyroid hormones. Additionally, as the NLR is affected by various diseases, it is necessary to consider other diseases concurrent with hyperthyroidism, which could influence systemic metabolism and organ system function. In the case of the hypertrophic cardiomyopathy phenotype that can often be accompanied in cats with hyperthyroidism, the NLR should be carefully interpreted, as a mean NLR value of 2.48 was reported in stage B hypertrophic cardiomyopathy cats without hyperthyroidism (Fries *et al.*, 2022).

Conclusions: The NLR, an inflammatory biomarker, offers valuable clinical insights that can guide therapeutic decisions and enhance the overall management of feline hyperthyroidism. This underscores the correlation between immune responses and the progressive nature of feline hyperthyroidism, and highlights the need for further studies to better understand and manage this complex endocrine disorder.

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Ethics approval: The work described in this manuscript involved the use of non-experimental (owned or unowned) animals. Established and internationally recognized high standards ('best practice') of veterinary clinical care for the individual cats were always followed. Ethical approval was obtained from the Institutional Animal Care and Use Committee [CBNUA-2007-22-01] of the Laboratory Animal Research Center of Chungbuk National University, and owners' consent was obtained in all cases.

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Authors contribution: YC and BTK designed the study. YC and TY collected and organized the data. YC and TY analyzed the data. YC drafted the manuscript. YC, TY, HK, and BTK revised the manuscript. BTK managed the resources and supervised the research. All authors read and approved the final manuscript.

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