STANDARD ARTICLE

Journal of Veterinary Internal Medicine ACVIM

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American College of
Veterinary Internal Medicine

Performance of lymph node cytopathology in diagnosis and characterization of lymphoma in dogs

Valeria Martini[1] | Giuseppe Marano[2] | Luca Aresu[3] | Ugo Bonfanti[4] |
Patrizia Boracchi[2] | Mario Caniatti[1] | Francesco Cian[5] | Matteo Gambini[1] |
Laura Marconato[6] | Carlo Masserdotti[7] | Arturo Nicoletti[3] | Fulvio Riondato[3] |
Paola Roccabianca[1] | Damiano Stefanello[1] | Erik Teske[8] | Stefano Comazzi[1]

[1]Department of Veterinary Medicine, University of Milan, Lodi, Italy

[2]Department of Clinical Sciences and Community Health, Laboratory of Medical Statistics, Biometry and Epidemiology "G.A. Maccaro", University of Milan, Milan, Italy

[3]Department of Veterinary Sciences, University of Turin, Grugliasco, Italy

[4]MYLAV La Vallonea Veterinary Diagnostic Laboratory, Rho MI, Italy

[5]Batt Laboratories, University of Warwick, Coventry, United Kingdom

[6]Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy

[7]IDEXX Laboratories, Milano, Italy

[8]Department of Clinical Sciences, Veterinary Faculty, Utrecht University, Utrecht, The Netherlands

Correspondence
Valeria Martini, Department of Veterinary Medicine, via dell'Università 6, 26900, Lodi, Italy.
Email: valeria.martini@unimi.it

Funding information
University of Milan

Abstract

Background: Cytopathology is a minimally invasive and convenient diagnostic procedure, often used as a substitute for histopathology to diagnose and characterize lymphoma in dogs.

Objectives: Assess the diagnostic performance of cytopathology in diagnosing lymphoma and its histopathological subtypes in dogs.

Animals: One-hundred and sixty-one lymph node samples from 139 dogs with enlarged peripheral lymph nodes.

Methods: Based only on cytopathology, 6 examiners independently provided the following interpretations on each sample: (a) lymphoma vs nonlymphoma; (b) grade and phenotype; and (c) World Health Organization (WHO) histopathological subtype. Histopathology and immunohistochemistry (IHC) findings were used as reference standards to evaluate diagnostic performance of cytopathology. Clinical, clinicopathologic, and imaging data also were considered in the definitive diagnosis.

Results: Classification accuracy for lymphoma consistently was >80% for all examiners, whereas it was >60% for low grade T-cell lymphomas, >30% for high grade B-cell lymphomas, >20% for high grade T-cell lymphomas, and <40% for low grade B-cell lymphomas. Interobserver agreement evaluated by kappa scores was 0.55 and 0.32 for identification of lymphoma cases, and of grade plus immunophenotype, respectively.

Conclusions and Clinical Importance: Cytopathology may result in accurate diagnosis of lymphoma, but accuracy decreases when further characterization is needed. Cytopathology represents a fundamental aid in identifying lymphoma and can be used as a screening test to predict grade and phenotype. However, these results

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must be confirmed using other ancillary techniques, including flow cytometry, histopathology, and immunohistochemistry (IHC).

KEYWORDS
accuracy, grade, morphology, phenotype

1 | INTRODUCTION

Lymphoma in dogs is a heterogeneous disease, encompassing many clinical presentations and morphological subtypes. The World Health Organization (WHO) classification of lymphoma in dogs is based on histopathology and immunohistochemistry (IHC).[1] In particular, marginal-zone lymphoma (MZL), follicular lymphoma (FL), mantle cell lymphoma, T-zone lymphoma (TZL), small lymphocytic lymphoma (SLL), lymphoplasmacytic lymphoma and T-cell rich B-cell lymphoma represent the indolent lymphoma subgroup,[2,3] whereas the other types form the aggressive lymphoma subgroup.[4] The distinction between indolent and aggressive lymphomas relies on the type of tumor growth within the nodal architecture (nodular vs diffuse), regardless of the number of mitoses observed, and therefore can be performed using histopathology only.[5] In dogs, identification of the WHO lymphoma subtype is relevant because clinical aggressiveness, prognostic factors and treatment response may vary among different histopathological subtypes.[4,5] Unfortunately, histopathology and IHC require invasive biopsy procedures and some time may elapse before a final diagnosis is obtained, thereby delaying lymphadenectomy. Conversely, cytopathology is less invasive and less time-consuming. Several morphological classifications have been proposed for lymphoma in dogs.[5,6] Among them, the updated Kiel scheme for cytopathology and the WHO classification for histopathology seem to be most appropriate, based on the veterinary literature.[7] Nodal architecture cannot be investigated by cytopathology, thus impeding the discrimination between indolent and aggressive lymphomas. The terms low-grade and high-grade are used by cytopathologists to predict clinical aggressiveness, but no standardized criteria are available for grade definition.[8]

Two studies have described the possible correlation between cytopathological and histopathological subtypes, matching the updated Kiel cytopathological classification system with the WHO histopathological scheme.[9,10] A previous study assessed intra- and interobserver agreement in the classification of lymphoma cases according to the updated Kiel classification.[9] However, currently, no large studies have been published analyzing the capability of cytopathology to potentially predict specific WHO lymphoma subtypes.

Our aim was to assess interobserver agreement and the predictive value of multiple cytopathologists for the diagnosis of (a) nodal lymphoma in dogs, (b) grade and immunophenotype, and (c) WHO histopathological subtype. The definitive diagnosis used as a standard for assessing the performance of the cytopathologists was made using histopathology and IHC findings, which were used as reference standards.
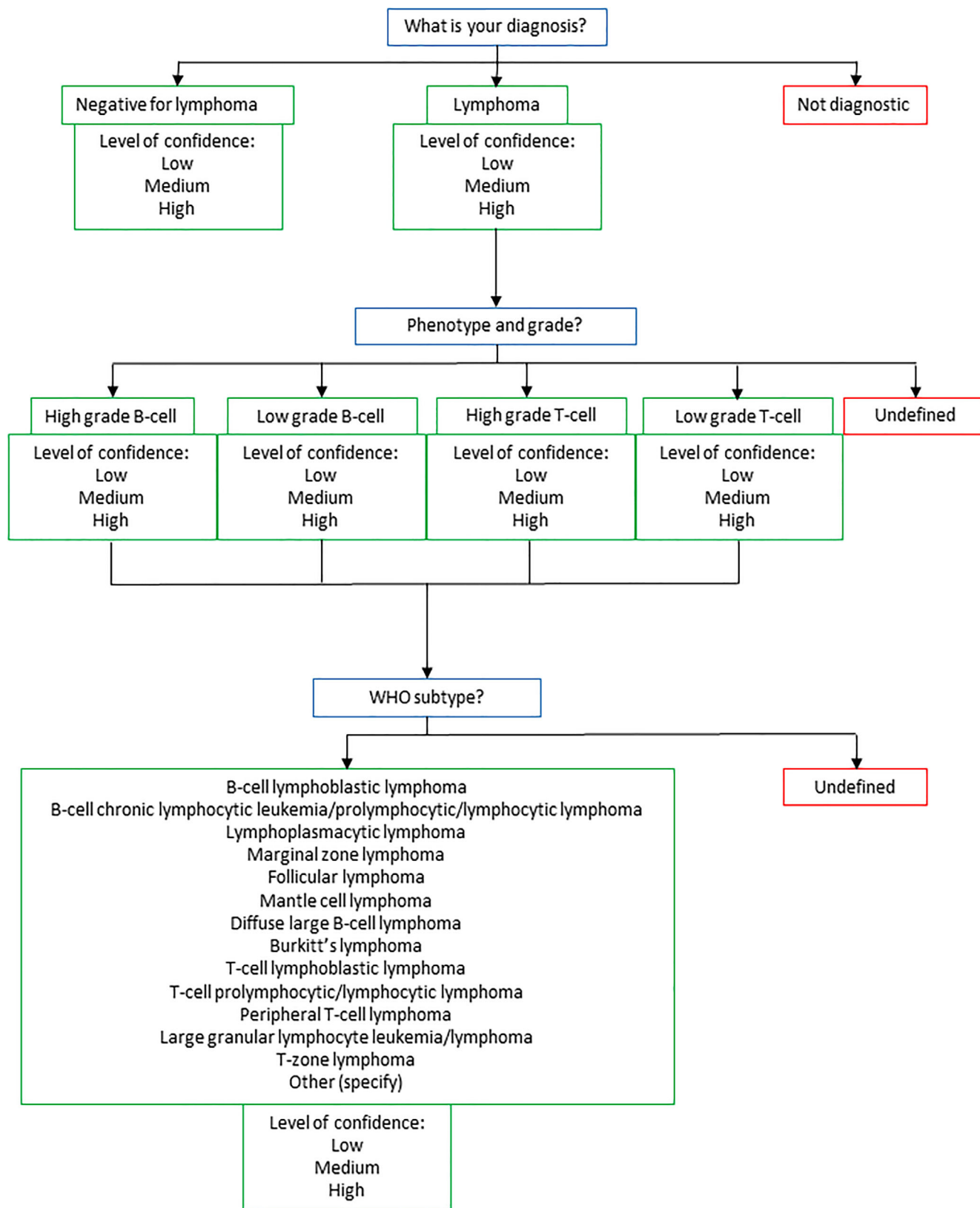
2 | MATERIALS AND METHODS

The databases of the diagnostic laboratories of the Veterinary Teaching Hospital of the University of Milan and of the University of Turin were retrospectively investigated and cases, for which enlargement of ≥1 peripheral lymph nodes (LN) was reported by the referring veterinarian, were selected. Cases were included if both cytopathological and histopathological results from the same enlarged peripheral LN were available, with lymphadenectomy having being performed within 10 days of cytopathology. Samples for both cytopathological and histopathological evaluation had to be available for review for cases to be included in the study.

Unstained glass smears obtained from fine needle aspirates and provided by the referring veterinarian were stained with May-Grünwald Giemsa and examined by a single operator (VM) to evaluate cellularity and overall quality of the smears. Cases were retained for the study when a minimum of 1 high-power field (60×) with intact cells was present, so as to exclude very poorly preserved and acellular samples. Samples included in the study were randomly assigned to all examiners blinded with regard to the final diagnoses.

All cytopathological samples included in the study were provided in 3 batches (50 to 62 slides in each batch) and evaluated independently by 6 examiners, including 4 board-certified clinical pathologists (European College of Veterinary Clinical Pathology, ECVCP), 1 board-certified anatomic pathologist (European College of Veterinary Pathology, ECVP), and 1 ECVP resident (UB, MC, FC, MG, CM, ET). For each sample, the examiners were required to fill out an online questionnaire, providing the following data (Figure 1): diagnosis (lymphoma, negative for lymphoma, not diagnostic), grade and phenotype (high grade B-cell, low grade B-cell, high grade T-cell, low grade T-cell, undefined), histopathological subtype according to the WHO classification of lymphoma in dogs (Table 1, other, undefined).[1] Whenever the diagnosis was "negative for lymphoma" or "not diagnostic," the examiner was not asked to further evaluate the sample. Similarly, the WHO histopathological subtype was not required if grade and phenotype were classified as "undefined." Examiners also had to provide their level of confidence (low, medium, or high) for each of the answers. The examiners did not confer before the beginning of the study and no guidelines were provided. Consequently, the 6 examiners were free to apply their own criteria and classify samples according to their experience in order to simulate what usually happens in veterinary diagnostic laboratories.

Histopathology and IHC were performed on formalin-fixed, paraffin-embedded LN sections according to published protocols, using anti-CD3 (clone F7.2.38), anti-CD5 (clone CD5/54/F6),

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FIGURE 1 Flowchart depicting the structure of the online questionnaire fulfilled by 6 examiners examining 161 canine lymph node aspirate cytological preparations

anti-CD79acy (clone HM57), and anti-CD20 (clone RB-9013-P) antibodies.[4] Lymphomas were classified according to the WHO criteria[1] by a single experienced anatomic pathologist (LA), who was aware of clinical data and results of any other tests performed, including hematology, phenotyping, and imaging. In case of uncertain WHO subtype, additional sections were cut, stained with hematoxylineosin, and reviewed by 2 additional anatomic pathologists (AN, PR). The definitive diagnosis was obtained by consensus.

2.1 | Statistical analysis

All 161 cytopathological samples were examined by the 6 examiners, for a total of 966 records. All data were entered into an electronic datasheet.

For the diagnosis of lymphoma and its grade and phenotype, classification accuracy was calculated for each modality of the 2 variables, defined as the proportion of cytopathological classifications that matched the definitive diagnosis divided by the total number of histopathological classifications. Interobserver agreement was estimated using the Fleiss kappa index (adequate for variables with >2 categories and multiple raters). The index was reported along with its respective 95% confidence interval (CI). The degree of agreement was deduced as previously described[11]: slight agreement for kappa values between 0.00 and 0.20, fair agreement for values between 0.21 and 0.40, moderate agreement for values between 0.41 and 0.60, substantial agreement for values between 0.61 and 0.80, and almost perfect agreement for values >0.80.

Concerning the evaluation of diagnostic accuracy, estimates of sensitivity and specificity and their respective 95% CI were calculated

TABLE 1 Summary of canine malignant lymphoma histopathological subtypes according to the World Health Organization classification scheme

| B-cell neoplasms |
| B-cell lymphoblastic lymphoma[a] |
| B-cell chronic lymphocytic leukemia/prolymphocytic/lymphocytic lymphoma[b] |
| Lymphoplasmacytic lymphoma[b] |
| Marginal zone lymphoma[b] |
| Follicular lymphoma[b] |
| Mantle cell lymphoma[b] |
| Diffuse large B-cell lymphoma[a] |
| Burkitt's lymphoma[a] |
| T-cell neoplasms |
| T-cell lymphoblastic lymphoma[a] |
| T-cell prolymphocytic/lymphocytic lymphoma[b] |
| Peripheral T-cell lymphoma[a] |
| Large granular lymphocyte leukemia/lymphoma[a] |
| T-zone lymphoma[b] |

Note: Primary extranodal lymphomas have not been listed.
[a]Aggressive (high grade) lymphoma.[4]
[b]Indolent (low grade) lymphoma.[2,3]

by means of a generalized estimating equation (GEE),[12] considering the correlation among the diagnoses made by the 6 examiners for each single biological sample (ie, within sample correlation). To that end, records considered "not diagnostic" or "undefined" were removed from the data. Sensitivity and specificity of the 4 modalities of the variable grade and phenotype (high grade B-cell, low grade B-cell, high grade T-cell, low grade T-cell) were calculated considering each modality as a single binary variable (eg, 1 for high grade B-cell lymphoma, 0 otherwise). Finally, for each modality of the 2 variables, the predictive accuracy of cytopathology was calculated by estimates of the expected positive predictive value (PPV) and negative predictive value (NPV) using a previously described method[13] and different values of prevalence from the published literature.[4,5,14]

To evaluate possible fluctuations in the diagnostic accuracy with the examiner's declared level of confidence, data were divided into 3 groups according to the level of confidence (high, medium, and low). Estimates of sensitivity and specificity were obtained as previously described, and separately for each subgroup.

Estimates of agreement and diagnostic accuracy could not be calculated reliably for WHO histopathological subtypes, because of the high number of modalities of the variable and the low frequency of several subtypes. Thus, descriptive analysis was reported, including cross-tabulation of cytopathological and definitive diagnoses and the proportion of biological samples univocally classified by at least 5 examiners.

All analyses were performed using the R software version 4.0.4[15] with the packages irrCAC[16] and geepack[17] added, and Knime Analytics Platform release 4.2.3.[18]

3 | RESULTS

A total of 161 cytopathological nodal samples obtained from 139 dogs were included in the study: 15 cases (9.3%) were definitively negative for lymphoma and were diagnosed as reactive nodal hyperplasia or metastatic infiltration by tumors other than lymphoma, whereas 146 (90.7%) were diagnosed as lymphoma. These included 75 diffuse large B-cell lymphomas (DLBC; 46.6%), 20 follicular lymphoma (FL; 12.4%), 19 marginal-zone lymphoma (MZL; 11.8%), 12 peripheral T-cell lymphoma (PTCL; 7.5%), 7 T-zone lymphoma (TZL; 4.3%), 7 Burkitt-like lymphoma (BLL; 4.3%), 3 B-cell small lymphocytic lymphoma (B-SLL; 1.9%), 2 B-cell lymphoblastic lymphoma (B-LBL; 1.2%), and 1 T-cell small lymphocytic lymphoma (T-SLL; 0.6%; Figure 2). Details on results of cytopathological evaluations are shown in Tables S1–S3.

3.1 | Cytopathological differentiation between lymphoma and nonlymphoma samples

A summary of the evaluations of each examiner is presented in Table 2. The classifications were homogeneous among the 6 examiners. As an example, the proportion of samples diagnosed as

lymphoma ranged from 82.0% to 85.1%, except for examiner #1. Fewer than 5% samples were considered not diagnostic. The estimate of the proportion of nondiagnostic samples, accounting for within sample correlation, was 4.0% (95% CI, 3.6%-4.4%). The classification accuracy of the diagnosis lymphoma also was homogeneous among examiners, ranging from 82.9% to 93.2%.

Moderate interobserver agreement was found, with a Fleiss kappa index of 0.55 (95% CI, 0.44-0.66) and 86.3% concordant diagnoses. In particular, 112 (69.6%) samples were unequivocally classified by all 6 examiners and 29 (18.0%) by 5 examiners.

Estimates of sensitivity and specificity accounting for within sample correlation are presented in Table 3, both being closed to 90%. Assuming prevalence of lymphoma was 51.6%,[14] the expected PPV and NPV were 90.3% and 91.9%, respectively. Assuming a prevalence of lymphoma of 95.2%,[5] the expected PPV and NPV were 99.4% and 37.9%.

3.2 | Lymphoma grade and phenotype

Only samples definitively diagnosed as lymphomas were considered in this second step analysis for a total of 146 biological samples (876 records), including 84 (57.5%) aggressive B-cell lymphoma, 42 (28.8%) indolent B-cell lymphoma, 12 (8.2%) aggressive T-cell and 8 (5.5%) indolent T-cell lymphoma.
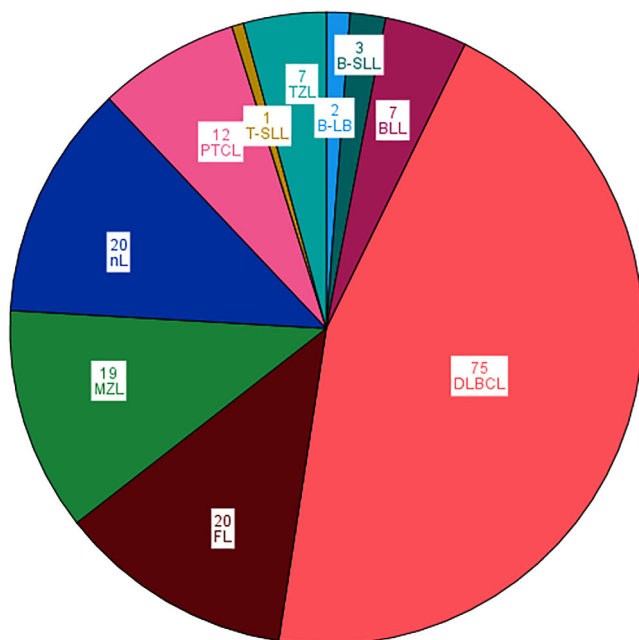
FIGURE 2   WHO histopathological classification of 161 canine nodal samples. B-LBL, B-cell lymphoblastic lymphoma; BLL, Burkitt-like lymphoma; B-SLL, B-cell small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LGL, large granular lymphocyte lymphoma; MZL, marginal zone lymphoma; nL, negative for lymphoma; PTCL, peripheral T-cell lymphoma; T-LBL, T-cell lymphoblastic lymphoma; T-SLL, T-cell small lymphocytic lymphoma; TZL, T-zone lymphoma

The classification accuracy of T-cell lymphoma, both high and low grade, ranged from 60% to 100%, except for high grade T-cell lymphoma for examiner #5 (Table 2, right panel). A lower classification accuracy was found for high grade B-cell lymphomas (range, 23.8%-77.4%). Finally, low grade B-cell lymphoma had the lowest classification accuracy, with almost all results <15%. The proportion of samples classified as undefined grade and phenotype by a single examiner accounted for up to 18.5% and the corresponding estimate, accounting for within sample correlation, was 16.5% (95% CI, 13.3%-20.4%).

Fair interobserver agreement was found, with a Fleiss kappa index of 0.32 (95% CI, 0.26-0.39) with 53.3% concordant diagnoses. In particular, 23 (15.8%) samples were unequivocally classified by all 6 examiners and 38 (26.0%) by 5 examiners.

Estimates of sensitivity and specificity accounting for within sample correlations are presented in Table 3. The lowest specificity estimate was found for high grade B-cell lymphomas and the lowest sensitivity estimate for low grade B-cell lymphomas. The sensitivity estimates for T-cell lymphomas of both high and low grades were affected by substantial uncertainty, as documented by the breadth of the corresponding 95% CI.

The expected PPV and NPV are shown in Figure 3. For the classification of high-grade B-cell lymphomas (Figure 3A), NPVs varied from 71.0% (for a prevalence of 44.4%) to 62.0% (for a prevalence of 54.4%). The expected NPVs overall are >80% for the classification of low-grade B-cell lymphomas (Figure 3B) and >90% for the classification of high-grade and low-grade T-cell lymphomas (Figure 3C,D). The PPVs were <70% for all classes.

3.3 | WHO lymphoma histopathological subtype

The frequency of WHO histopathological subtypes diagnosed by each examiner is shown in Tables 4 and S4. The percentage of correct diagnoses was consistently >65% and >75% for DLBCL and TZL, respectively (except for examiner #5 for both diagnoses), whereas it never exceeded 60% for MZL and was consistently <40% for PTCL.

Sixty-one (41.8%) samples were unequivocally classified by at least 5 examiners, including 29 samples with a cytopathological diagnosis of DLBCL and 21 samples in which the examiners did not define WHO subtype.

3.4 | Level of confidence

When diagnosing lymphoma vs nonlymphoma, the level of confidence was high in 703 (75.6%) records, medium in 148 (15.9%), and low in 79 (8.5%). When diagnosing lymphoma grade and phenotype, level of confidence was high in 300 (39.5%) records, medium in 314 (41.3%) and low in 146 (19.2%). When diagnosing WHO histopathological subtype, level of confidence was high in 200 (27.7%) records, medium in 298 (41.3%) and low in 223 (30.9%).

Estimates of sensitivity and specificity according to the degree of confidence of the examiner (low, medium, high) are presented in

TABLE 2 Cytopathological diagnosis of lymphoma and its grade and phenotype given by 6 examiners

Examiner	Lymphoma diagnosis (N = 161)				Lymphoma grade and phenotype (N = 146)			
	Classification	N (%)	Correct diagnoses (%)	Classification accuracy (%)	Classification	N (%)	Correct diagnoses (%)	Classification accuracy (%)
1	Lymphoma	121 (75.2)	121/121 (100.0)	121/146 (82.9)	HG B-cell	57 (39.0)	40/57 (70.2)	40/84 (47.6)
	Nonlymphoma	33 (20.5)	14/33 (42.4)	14/15 (93.3)	LG B-cell	16 (11.0)	5/16 (31.3)	5/42 (11.9)
	Not diagnostic	7 (4.3)			HG T-cell	40 (27.4)	9/40 (22.5)	9/12 (75.0)
					LG T-cell	6 (4.1)	6/6 (100.0)	6/8 (75.0)
					Undefined	27 (18.5)		
2	Lymphoma	134 (83.2)	133/134 (99.3)	133/146 (91.1)	HG B-cell	89 (61.0)	65/89 (73.0)	65/84 (77.4)
	Nonlymphoma	20 (12.4)	14/20 (70.0)	14/15 (93.3)	LG B-cell	6 (4.1)	1/6 (16.7)	1/42 (2.4)
	Not diagnostic	7 (4.3)			HG T-cell	25 (17.1)	8/25 (32.0)	8/12 (66.7)
					LG T-cell	8 (5.5)	5/8 (62.5)	5/8 (62.5)
					Undefined	18 (12.3)		
3	Lymphoma	137 (85.1)	135/137 (98.5)	135/146 (92.5)	HG B-cell	88 (60.3)	62/88 (70.5)	62/84 (73.8)
	Nonlymphoma	18 (11.2)	12/18 (66.7)	12/15 (80.0)	LG B-cell	7 (4.8)	5/7 (71.4)	5/42 (11.9)
	Not diagnostic	6 (3.7)			HG T-cell	29 (19.9)	9/29 (31.0)	9/12 (75.0)
					LG T-cell	9 (6.2)	7/9 (77.8)	7/8 (87.5)
					Undefined	13 (8.9)		
4	Lymphoma	137 (85.1)	136/137 (99.3)	136/146 (93.2)	HG B-cell	75 (51.4)	55/75 (73.3)	55/84 (65.5)
	Nonlymphoma	18 (11.2)	13/18 (72.2)	13/15 (86.7)	LG B-cell	9 (6.2)	5/9 (55.6)	5/42 (11.9)
	Not diagnostic	6 (3.7)			HG T-cell	29 (19.9)	9/29 (31.0)	9/12 (75.0)
					LG T-cell	9 (6.2)	7/9 (77.8)	7/8 (87.5)
					Undefined	24 (16.4)		
5	Lymphoma	133 (82.6)	128/133 (96.2)	128/146 (87.7)	HG B-cell	32 (21.9)	20/32 (62.5)	20/84 (23.8)
	Nonlymphoma	23 (14.3)			LG B-cell	47 (32.2)	17/47 (36.2)	17/42 (40.5)
	Not diagnostic	5 (3.1)	9/23 (39.1)	9/15 (60.0)	HG T-cell	22 (15.1)	4/22 (18.2)	4/12 (33.3)
					LG T-cell	27 (18.5)	6/27 (22.2)	6/8 (75.0)
					Undefined	18 (12.3)		
6	Lymphoma	132 (82.0)	132/132 (100.0)	132/146 (90.4)	HG B-cell	87 (59.6)	61/87 (70.1)	61/84 (72.6)
	Nonlymphoma	23 (14.3)	15/23 (65.2)	15/15 (100.0)	LG B-cell	6 (4.1)	4/6 (66.7)	4/42 (9.5)
	Not diagnostic	6 (3.7)			HG T-cell	19 (13.0)	8/19 (42.1)	8/12 (66.7)
					LG T-cell	10 (6.8)	8/10 (80.0)	8/8 (100.0)
					Undefined	24 (16.4)		

Note: Correct diagnoses are the proportions of cytopathological diagnoses confirmed via histopathology and immunohistochemistry. Classification accuracy is the proportion of histopathological diagnoses correctly classified by the examiner.

Abbreviations: HG, high grade; LG, low grade.

Table 5. Concerning the diagnosis of lymphoma, the estimates of sensitivity increased with increasing level of confidence, whereas the estimates of specificity did not, as documented by the overlapping 95% CI. The estimate of sensitivity for the diagnosis of high grade B-cell lymphoma increased with increasing level of confidence, whereas the estimate of specificity decreased. The opposite was observed for low grade B-cell lymphomas. Finally, based on the large and overlapping 95% CI, no evidence emerged supporting variations of sensitivity and specificity estimates with level of confidence for the diagnosis of T-cell lymphomas, both high and low grade.

4 | DISCUSSION

In dogs, lymphoma diagnosis is obtained by means of different tests, and several classifications have been proposed accordingly. The WHO classification is broadly used, but lymphadenectomy is required for this classification. To our knowledge, whether histopathological subtype can be predicted by cytopathology has been assessed in 1 study only, in which the updated Kiel classification was applied by 2 examiners.[9] In our study, 161 nodal cytopathological samples were evaluated by 6 independent examiners and results were compared with the WHO classification diagnosis obtained from the same lymph node

TABLE 3 Estimates of sensitivity and specificity of cytopathological diagnosis of lymphoma and its grade and phenotype, using histopathology and immunohistochemistry results as gold standard

	Sensitivity estimate (95% CI) (%)	Specificity estimate (95% CI) (%)
Lymphoma	92.6 (88.8-95.1)	89.4 (79.0-95.0)
High grade B-cell lymphoma	68.8 (62.7-74.3)	60.9 (51.2-69.8)
Low grade B-cell lymphoma	17.9 (12.3-25.4)	90.1 (86.6-92.7)
High grade T-cell lymphoma	72.9 (57.4-84.3)	82.5 (77.6-86.4)
Low grade T-cell lymphoma	82.2 (59.1-93.6)	95.4 (93.2-97.0)

using a combination of clinical data, clinicopathologic data, and imaging results, histopathology and IHC. Because of the disappointing intra- and interobserver agreement reported for the updated Kiel classification,[9] in our study examiners were not required to apply it, but they were asked to classify lymphoma samples based on their own criteria and experience. Cytopathologists showed high performance in identifying lymphoma. However, performance was lower when a more detailed characterization was required.

We observed high sensitivity and specificity estimates, and moderate interobserver agreement in diagnosing lymphoma by cytopathology. This finding was expected because most lymphomas in our dataset and in the published literature are composed of large cells,[1-7,9,10] which represent only a minority of the lymphoid population in processes other than lymphoma. We also calculated PPV and NPV values for different levels of prevalence. Both PPV and NPV were >90% when a prevalence of 54% was considered, which has been reported as the prevalence of lymphomas among nodal aspirates made for any reason.[14] In this scenario, both lymphoma and non-lymphoma cytopathological diagnoses are likely to be confirmed by histopathology. Conversely, NPV decreased markedly when a lymphoma prevalence of 95% was considered (prevalence of lymphoma diagnoses when lymphoma is clinically suspected).[5] This finding is not surprising in the context of statistical analysis, because for constant sensitivity and specificity, NPV decreases with increasing prevalence. From a practical point of view, this observation means that if lymphoma is suspected clinically, negative cytopathological results are less trustworthy.

Although rare, both false positive and false negative results were obtained. In the clinical setting, this may lead to 2 different scenarios. A false negative lymphoma result may prompt the clinician toward further laboratory tests to define the cause of lymphadenomegaly, including molecular tests and serology for infectious and parasitic diseases, simply resulting in a delayed lymphoma diagnosis. Conversely, a false positive result is a serious diagnostic error and may lead to unnecessary treatment or euthanasia. Therefore, if the clinical presentation does not support a cytopathological diagnosis of lymphoma,

confirmatory tests must be performed, including flow cytometry (FC), PCR for antigen receptor rearrangement (PARR), or histopathology and IHC. A definitive diagnosis of lymphoma should only be made combining clinical information with results obtained by a combination of the aforementioned tests.

When the performance of cytopathologists in lymphoma characterization was investigated, results were more disappointing. In fact, only fair agreement among examiners was found, as further demonstrated by the heterogeneity of classifications provided by the examiners. As an example, the proportion of samples classified as high grade B-cell lymphomas varied from 21.9% to 61.0% (Table 2). Higher NPV than PPV estimates were obtained for almost all grade and phenotype groups within the range of prevalence reported in the literature. All of the groups were considered as binary variables (eg, low grade T-cell vs otherwise), which is crucial to interpret predictive value estimates. As an example, if a low-grade T-cell lymphoma is diagnosed by cytopathology, the probability that it will be confirmed by histopathology ranges from 55% to 70% (depending on the prevalence). On the contrary, if a low grade T-cell lymphoma is excluded by cytopathology, it has a probability close to 100% of being excluded by histopathology also. The same applies for the remaining groups, except for high grade B-cell lymphoma when the prevalence is >51%. Whenever high grade B-cell lymphoma represents >51% of the population of interest, positive cytopathological results are more reliable than negative ones. Still, the pretest probability of having a specific lymphoma subtype (in terms of grade and phenotype) relies on the prevalence of that subtype not only in the general population of dogs with lymphoma, but also in dogs of that specific breed. Thus, we also present the predictive values for the entire 0% to 100% ranges of prevalence (or pretest probability) so as to provide data usable in any clinical setting. As expected, for each lymphoma subgroup, increasing PPV and decreasing NPV were found with increasing prevalence. As an example, if a dog has an 80% pretest probability of having high grade B-cell lymphoma, the PPV will be close to 90%, and the NPV <40%. Interestingly, optimal PPV and NPV are reached for low grade T-cell lymphoma even with <50% pretest probability.

No more than 70% of the suspected DLBCLs were confirmed, and the percentage of correct diagnoses was much lower for other B-cell histopathological subtypes (Table 4). For all examiners, the misdiagnosis was mainly correlated with a wrong determination of lymphoma grade, rather than phenotype (Table S4), as documented by the fact that most cases were definitively diagnosed with another B-cell lymphoma WHO histopathological subtype. These findings have several explanations.

First, the reported high frequency of DLBCL in dogs may have led to overestimating this diagnosis compared with other less common subtypes. Second, transformation of FL into DLBCL and MZL has been described previously in dogs, complicating the cytopathological diagnosis. In fact, both histopathological subtypes are characterized by loss of the nodal follicular pattern and an increasing number of large cells (ie, centroblasts),[2] thus making evaluation of nodal architecture crucial in these cases. Third, the challenge in recognizing the grade of B-cell lymphomas may be a consequence of the lack of
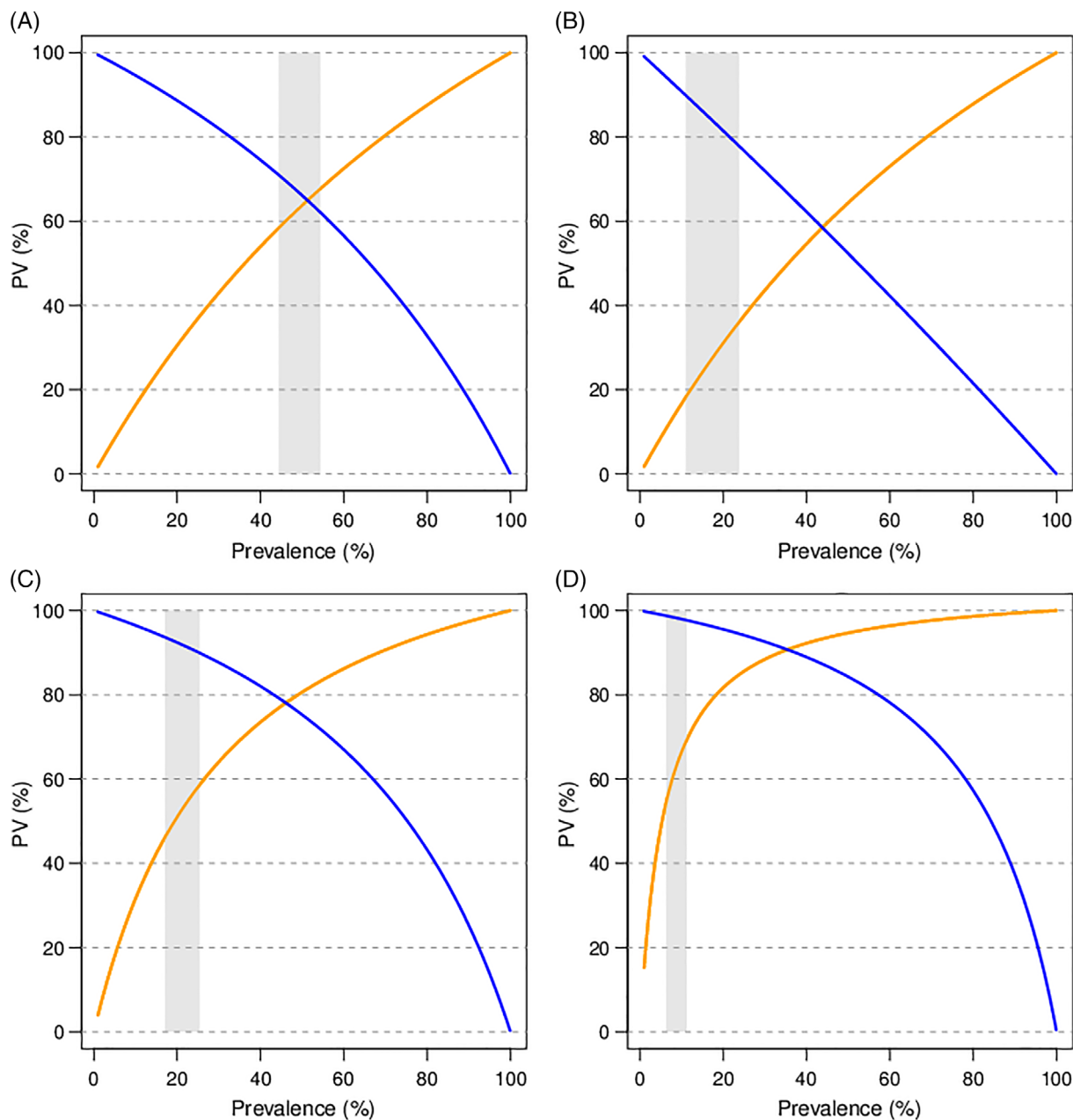
FIGURE 3 Expected predictive values of cytopathological classification of canine nodal lymphoma, based on the sensitivity and specificity values reported in Table 3. Orange lines: positive predictive value (PPV). Blue lines: negative predictive value (NPV). Gray-shaded areas represent the prevalence range reported in the literature (see methods section for details). (A) PPV and NPV for the identification of high grade B-cell lymphoma. (B) PPV and NPV for the identification of low grade B-cell lymphoma. (C) PPV and NPV for the identification of high grade T-cell lymphoma. (D) PPV and NPV for the identification of low grade T-cell lymphoma. PV, predictive value

classification criteria shared among examiners. Indeed, they may have indicated high or low grade based on cell morphology, or on the number of mitoses per field, or to predict the expected clinical course.[8] This is particularly relevant for MZL, which are indolent lymphomas, with a low mitotic index, but often with aggressive clinical behavior in dogs diagnosed at an advanced stage.[19] Being aware of these latter features, cytopathologists might have diagnosed high grade B-cell lymphoma, which would have been considered discordant from the histopathological diagnosis.

Regarding T-cell lymphomas, the highest percentages of correct diagnoses were found for TZL (Table 4). Cytopathologically, TZL cells have a unique morphology[20] and the main differential diagnosis is nodal paracortical hyperplasia. In our dataset, 2 cases of nodal reactive hyperplasia were erroneously diagnosed as TZL by cytopathologists (Table S3, cases #43 and #104, examiner #5). Misclassification with other lymphoma subtypes occurred, but the opportunity to use FC in conjunction with cytopathology may enhance the chance to make a correct diagnosis in these cases,

TABLE 4 Cytopathological diagnosis of canine lymphoma WHO histopathological subtype by 6 examiners

Subtype classification	Number of samples (correct diagnoses [N, %])					
	Examiner 1	Examiner 2	Examiner 3	Examiner 4	Examiner 5	Examiner 6
DLBCL	55 (37, 67.3%)	73 (49, 67.1%)	83 (54, 65.1%)	75 (52, 69.3%)	27 (14, 51.9%)	57 (40, 70.2%)
BLL		5 (1, 20.0%)			3 (0, 0.0%)	9 (1, 11.1%)
B-LBL		7 (0, 0.0%)	1 (0, 0.0%)		4 (1, 25.0%)	
FL		1 (0, 0.0%)			15 (1, 6.7%)	
MZL	13 (4, 30.8%)	5 (2, 40.0%)	5 (3, 60.0%)	9 (5, 55.6%)	11 (2, 18.2%)	5 (3, 60.0%)
B-SLL		3 (0, 0.0%)	4 (0, 0.0%)		8 (0, 0.0%)	1 (0, 0.0%)
PTCL	37 (9, 24.3%)	22 (8, 36.4%)	26 (8, 30.8%)	30 (9, 30.0%)	22 (3, 13.6%)	12 (4, 33.3%)
TZL	7 (6, 85.7%)	3 (3, 100.0%)	6 (5, 83.3%)	4 (4, 100.0%)	17 (5, 29.4%)	8 (6, 75.0%)
T-SLL		5 (0, 0.0%)	3 (0, 0.0%)	2 (0, 0.0%)	8 (0, 0.0%)	
LGL	1 (0, 0.0%)				1 (0, 0.0%)	
Lymphoplasmacytic	1 (0, 0.0%)				9 (0, 0.0%)	
Mantle-cell					5 (0, 0.0%)	
Plasmacytoid						1 (0, 0.0%)
T-LBL	1 (0, 0.0%)	3 (0, 0.0%)	3 (0, 0.0%)		3 (0, 0.0%)	2 (0, 0.0%)
Nonlymphoma	33 (14, 42.4%)	20 (14, 70.0%)	18 (12, 66.7)	18 (13, 72.2%)	23 (9, 39.1%)	23 (15, 65.2%)
Lymphoma, undefined WHO subtype	13	14	12	23	5	43

Note: Correct diagnoses are the proportions of samples in which the cytopathological diagnosis was confirmed via histopathology and immunohistochemistry. These results are reported for descriptive purposes, and should not be used for evaluating predictive ability of the cytopathological method because of the low prevalence of different WHO subtypes.

Abbreviations: B-LBL, B-cell lymphoblastic lymphoma; BLL, Burkitt-like lymphoma; B-SLL, B-cell Small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LGL, large granular lymphocyte lymphoma; MZL, marginal zone lymphoma; PTCL, peripheral T-cell lymphoma; T-LBL, T-cell lymphoblastic lymphoma; T-SLL, T-cell small lymphocytic lymphoma; TZL, T-zone lymphoma.

TABLE 5 Estimates of sensitivity and specificity of cytopathological diagnosis of lymphoma and its grade and phenotype, using histopathology and immunohistochemistry results as gold standard, according to the level of confidence of the examiners

	Level of confidence	Sensitivity estimate (95% CI) (%)	Specificity estimate (95% CI) (%)
Lymphoma	Low	69.2 (56.4-79.5)	66.7 (34.5-88.3)
	Medium	82.7 (74.1-88.9)	81.0 (48.0-95.2)
	High	97.5 (94.6-98.9)	93.2 (74.3-98.5)
High grade B-cell lymphoma	Low	31.9 (23.2, 42.0)	82.1 (68.8, 90.5)
	Medium	60.1 (51.1, 68.4)	67.7 (57.6, 76.3)
	High	92.8 (85.7, 96.5)	37.1 (24.0, 52.4)
Low grade B-cell lymphoma	Low	35.8 (21.2, 53.8)	72.6 (63.1, 80.4)
	Medium	21.9 (13.8, 32.9)	90.7 (85.2, 94.3)
	High	3.1 (0.4, 18.9)	98.7 (95.9, 99.6)
High grade T-cell lymphoma	Low	64.2 (42.9, 81.1)	72.4 (64.1, 79.4)
	Medium	74.2 (57.4, 85.9)	73.8 (66.7, 79.9)
	High	69.5 (38.1, 89.4)	95.5 (90.5, 97.9)
Low grade T-cell lymphoma	Low	59.7 (26.8, 85.8)	89.1 (82.7, 93.4)
	Medium	81.6 (45.7, 95.9)	96.0 (92.6, 97.9)
	High	86.6 (43.1, 98.2)	98.4 (95.7, 99.4)

relying on the typical loss of expression of the pan-leukocyte marker CD45.[21]

Based on cytopathology, PTCL was the most frequently diagnosed T-cell subtype, but <40% of the cases were confirmed.

Interestingly, for all examiners, >25% samples diagnosed with PTCL finally were classified as DLBCL. Based on histopathology, DLBCL and PTCL share some morphological aspects because both types are composed of medium to large sized cells and show a diffuse growth

pattern. Nevertheless, PTCL cells rarely have nucleoli and have a more variable cell size. Moreover, hyperplasia of postcapillary venules is common. Immunohistochemistry is considered mandatory to discriminate between these 2 histopathological subtypes.[5] Inclusion of phenotyping techniques (FC or IHC) may increase the accuracy of cytopathology in identifying PTCL.

The examiners in our study were asked to provide the confidence level for each diagnosis. According to the results, the examiners were confident in discriminating between lymphomas and nonlymphomas whereas they reported difficulties in predicting grade and phenotype or WHO histopathological subtype by cytopathology (Tables S1–S3).

We calculated sensitivity and specificity of the cytopathological diagnoses according to the level of confidence. Concerning the diagnosis of lymphoma, both increased with increasing confidence, suggesting that if cytopathologists are confident with their diagnosis, the diagnostic performance of the test itself improves. Modifiers should be included in the cytopathological reports to alert clinicians to consider other diagnostic tests when a low confidence diagnosis of lymphoma is made.[22] For high grade B-cell lymphomas, increasing levels of confidence resulted in a lower prevalence of false negative results (higher sensitivity) and a higher prevalence of false positive results (lower specificity). This latter finding is difficult to interpret but may be caused by the fact that cytopathologists are more comfortable in diagnosing high grade B-cell lymphomas, based on the reported higher frequency of this lymphoma subtype in dogs. Once more, our results confirm the need of further tests for lymphoma classification, regardless of the level of confidence of the cytopathologists.

Our study had some limitations. First, the number of non-lymphoma samples was low. Indeed, clinicians tend not to remove nodes if lymphoma is not suspected by cytopathology. Second, separate statistical analyses for suspected grade and phenotype were not performed because the examiners were asked to choose across 4 combinations (Figure 1, second step). Nevertheless, all examiners claimed that they were more confident in predicting grade than phenotype. Third, multiple cytological preparations were available for a few cases. However, the examiners were not aware of this and were instructed to consider each cytological preparation independently. In addition, alternative estimates of diagnostic accuracy were calculated using methods accounting for multiway clustering[23] and no substantial difference with the estimates reported in Tables 3 and 5 was found. Multiple slides (rather than 1 only) usually are submitted to the laboratory combined with clinical information, and cytopathologists may require immunophenotyping, thereby improving diagnostic performance. In our study, only 1 smear was available, and all examiners were blinded to clinical information and phenotype data, which represents a major discrepancy with daily laboratory practice. Finally, decreased numbers of some WHO subtypes, reflecting their low prevalence in the canine population, prevented us from calculating their predictive values. Therefore, only descriptive statistics were reported.

In conclusion, in our study, cytopathologists accurately diagnosed lymphomas, but their performance decreased when further characterization, including phenotype and grade, was attempted. Thus, cytopathology represents a fundamental aid in identifying lymphoma and

cytopathology that can be used as a screening test to predict grade and phenotype, but these data must be confirmed using other ancillary techniques, including FC and histopathology in combination with IHC.

CONFLICT OF INTEREST DECLARATION
Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION
Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION
All dogs were privately owned and sampled for diagnostic purposes with informed consent of the owners. Thus, according to the regulations of the University of Milan (EC decision October 29, 2012, renewed with protocol n°02-2016) and of the University of Turin (prot. n. 1965-2017), a specific approval of the Ethical Committee was not required for research use of the surplus specimens.

HUMAN ETHICS APPROVAL DECLARATION
Authors declare human ethics approval was not needed for this study.

ORCID
Valeria Martini https://orcid.org/0000-0002-9882-2867
Luca Aresu https://orcid.org/0000-0002-7893-1740
Laura Marconato https://orcid.org/0000-0002-7843-615X
Damiano Stefanello https://orcid.org/0000-0003-2726-0366

REFERENCES
1. Valli VE, San Myint M, Barthel A, et al. Classification of canine malignant lymphomas according to the World Health Organization criteria. *Vet Pathol.* 2011;48(1):198-211.
2. Valli VE, Vernau W, de Lorimier LP, Graham PS, Moore PF. Canine indolent nodular lymphoma. *Vet Pathol.* 2006;43:241-256.
3. Flood-Knapik KE, Durham AC, Gregor TP, Sánchez MD, Durney ME, Sorenmo KU. Clinical, histopathological and immunohistochemical characterization of canine indolent lymphoma. *Vet Comp Oncol.* 2012;11(4):272-286.
4. Aresu L, Martini V, Rossi F, et al. Canine indolent and aggressive lymphoma: clinical spectrum with histologic correlation. *Vet Comp Oncol.* 2015;13(4):348-362.
5. Valli VE, Kass PH, San Myint M, et al. Canine lymphomas: association of classification type, disease stage, tumor subtype, mitotic rate, and treatment with survival. *Vet Pathol.* 2013;50(5):738-748.
6. Ponce F, Magnol JP, Ledieu D, et al. Prognostic significance of morphological subtypes in canine malignant lymphomas during chemotherapy. *Vet J.* 2004;167(2):158-166.
7. Sayag D, Fournel-Fleury C, Ponce F. Prognostic significance of morphotypes in canine lymphomas: a systematic review of literature. *Vet Comp Oncol.* 2018;16(1):12-19.

8. Avallone G, Rasotto R, Chambers JK, et al. Review of histological grading systems in veterinary medicine. *Vet Pathol*. 2021;58(5):809-828.

9. Teske E, van Heerde P. Diagnostic value and reproducibility of fine needle aspiration cytology in canine malignant lymphoma. *Vet Q*. 1996;18(3):112-115.

10. Ponce F, Marchal T, Magnol JP, et al. A morphological study of 608 cases of canine malignant lymphoma in France with a focus on comparative similarities between canine and human lymphoma morphology. *Vet Pathol*. 2010;47(3):414-433.

11. Landis RJ, Koch GG. The measurement of observer agreement for categorical data. *Biometrics*. 1977;33:159-174.

12. Genders TS, Spronk S, Stijnen T, et al. Methods for calculating sensitivity and specificity of clustered data: a tutorial. *Radiology*. 2012;265(3):910-916.

13. Hayden SR, Brown MD. Likelihood ratio: a powerful tool for incorporating the results of a diagnostic test into clinical decision making. *Ann Emerg Med*. 1999;33(5):575-580.

14. Amores Fuster I, Cripps P, Graham P, Marrington AM, Blackwood L. The diagnostic utility of lymph node cytology samples in dogs and cats. *J Small Anim Pract*. 2015;56(2):125-129.

15. R Core Team R: A Language and Environment for Statistical Computing; 2021. https://www.R-project.org/. Accessed 01 July 2021.

16. Kilem LG irrCAC: Computing Chance-Corrected Agreement Coefficients (CAC). R package v1.0; 2019. https://CRAN.R-project.org/package=irrCAC. Accessed 01 July 2021.

17. Højsgaard S, Halekoh U, Yan J. The R package geepack for generalized estimating equations. *J Stat Softw*. 2005;15(1):1-11.

18. Berthold MR, Cebron N, Dill F, et al. KNIME: the Konstanz Information Miner. In: Preisach C, Burkhardt H, Schmidt-Thieme L, Decker R, eds. *Data Analysis, Machine Learning and Applications: Proceedings of the 31st Annual Conference of the Gesellschaft Für Klassifikation EV, March 7-9*. Albert-Ludwigs-Universität Freiburg: Springer Science and Business Media; 2007.

19. Cozzi M, Marconato L, Martini V, et al. Canine nodal marginal zone lymphoma: descriptive insight into the biological behavior. *Vet Comp Oncol*. 2018;16(2):246-252.

20. Fournel-Fleury C, Ponce F, Felman P, et al. Canine T-cell lymphomas: a morphological, immunological, and clinical study of 46 new cases. *Vet Pathol*. 2002;39(1):92-109.

21. Martini V, Poggi A, Riondato F, Gelain ME, Aresu L, Comazzi S. Flow-cytometric detection of phenotypic aberrancies in canine small clear cell lymphoma. *Vet Comp Oncol*. 2015;13(3):281-287.

22. Christopher MM, Hotz CS. Cytologic diagnosis: expression of probability by clinical pathologists. *Vet Clin Pathol*. 2004;33(2):84-95.

23. Zeileis A, Köll S, Graham N. Various versatile variances: an object-oriented implementation of clustered covariances in R. *J Stat Softw*. 2020;95(1):1-36.

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