

ORIGINAL RESEARCH

High diagnostic accuracy of the Sysmex XT-2000iV delta total nucleated cells on effusions for feline infectious peritonitis

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Key Words

Cat, coronavirus, likelihood ratio, Rivalta's test, sensitivity, specificity

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Background: The Δ WBC (the ratio between DIFF and BASO counts of the Sysmex XT-2000iV), hereafter defined as Δ TNC (total nucleated cells), is high in effusions due to feline infectious peritonitis (FIP), as cells are entrapped in fibrin clots formed in the BASO reagent. Similar clots form in the Rivalta's test, a method with high diagnostic accuracy for FIP.

Objectives: The objective of this study was to determine the diagnostic accuracy for FIP and the optimal cutoff of Δ TNC.

Methods: After a retrospective search of our database, DIFF and BASO counts, and the Δ TNC from cats with and without FIP were compared to each other. Sensitivity, specificity, and positive and negative likelihood ratios (LR+, LR-) were calculated. A ROC curve was designed to determine the cutoff for best sensitivity and specificity.

Results: Effusions from 20 FIP and 31 non-FIP cats were analyzed. The Δ TNC was higher ($P < .001$), and BASO and DIFF counts were lower ($P < .001$ and $P < .05$) in FIP than in non-FIP cats. Only 2 FIP cats with atypical effusions had a Δ TNC < 3.0 . The cutoff identified by the ROC curve (area under curve: 0.94; $P < .001$) was 1.7 (Sensitivity = 90.0%; Specificity = 93.53%; LR+ = 13.9; LR- = 0.1). A Δ TNC > 2.5 had 100% specificity.

Conclusions: The Δ TNC has a high diagnostic accuracy for FIP-related effusions by providing an estimate of precipitable proteins, as the Rivalta's test, in addition to the cell count. As fibrin clots result in false lower BASO counts, the Δ TNC is preferable to the WBC count generated by the BASO channel alone in suspected FIP effusions.

Introduction

Feline infectious peritonitis (FIP) is an ubiquitous lethal disease caused by the feline coronavirus (FCoV) and is triggered by an excessive immune response of cats infected with mutated FCoV variants.¹

The ante-mortem diagnosis of FIP is always challenging, especially in its noneffusive ("dry") form, due to the variable clinical signs and the poor specificity of many laboratory assays. Among these, serum protein electrophoresis and the α 1-acid glycoprotein (AGP) measurement may support a clinical suspicion of FIP.²⁻⁶ However, neither of these tests provides a definitive diagnosis of FIP.² On the other hand, the effusive ("wet") form is easier to diagnose,

based on the signalment and history, the results of the biochemical tests mentioned above, and especially the analysis of effusions.

Macroscopically, the typical FIP effusion is yellow, turbid, viscous, and often contains fibrin strands. The protein content is usually high with a decreased albumin to globulin ratio.⁷ Cell counts range from $2-6 \times 10^9/L$, sometimes up to $30 \times 10^9/L$,⁸ and the cytologic pattern, which is only highly suggestive but not definitely diagnostic for FIP, consists mostly of nondegenerate neutrophils, macrophages, lymphocytes, and rare plasma cells in a proteinaceous background.¹

Conversely, the Rivalta's test has been recently proposed as a test with high accuracy for the diagnosis

of FIP.⁹ The Rivalta's test is an inexpensive, easy to perform assay, used to differentiate transudates from exudates. The positive reaction to the acetic acid is due to the presence of a high concentration of proteins, including fibrinogen and other acute phase proteins which visibly clot upon contact with acids.¹⁰ These components are particularly abundant in effusions from cats with FIP, but can also be increased in effusions due to bacterial peritonitis and pleuritis or lymphoma.

Therefore in feline medicine, the Rivalta's test coupled with cytology of the effusion may be a quick way to distinguish FIP effusions from other types of effusions. Several studies demonstrated the diagnostic utility of Rivalta's test for FIP based on its high sensitivity and accuracy.⁹

In a recent study on canine and feline effusions it was shown that the Delta (Δ) TNC (the ratio between total nucleated cell counts [TNCC] in the DIFF and BASO channel of the laser counter Sysmex XT-2000iV, reported by the instrument as Δ WBC) is higher in effusions of cats affected by FIP than in other effusions.¹¹ The BASO channel uses an acidic reagent that induces the collapse of the cells, except for basophils. In FIP effusions, this reagent induces also the formation of clots entrapping the cells and resulting in a falsely low BASO count. Therefore, this mechanism, responsible for the increase in the Δ TNC, is very similar to the analytic principle of the Rivalta's test.

The aim of this study was to determine the diagnostic accuracy of the Δ TNC for FIP effusions according to the Standards for Reporting of Diagnostic Accuracy (STARD) approach^{12,13} in a larger number of cases and to assess whether it may have the same diagnostic utility as that reported for the Rivalta's test⁹, and to define the cutoff value of Δ TNC minimizing false-positive and -negative results for the diagnosis of FIP.

Material and Methods

Retrospective selection of cases

This was a retrospective study performed on data from effusion specimens submitted to the Department of Veterinary Science and Public Health of the University of Milan (DIVET) within our routine diagnostic activity and collected under informed consent of the owners. The database of DIVET was searched to select feline intracavitary effusions analyzed with the Sysmex laser counter for the period from June 2009 to June 2013. Cases included for data analysis were selected by the following inclusion and exclusion criteria:

Inclusion criteria:

- (1) Availability of complete documentation of physico-chemical analysis of the effusion, including specific gravity and protein content, estimated by refractometric analysis.
- (2) Availability of extensive information about the final diagnosis according to the criteria described below.
- (3) Availability of cyto-centrifuged slides in the archive of DIVET to re-assess the cytologic pattern of effusions in cases where no cytologic report was available in the database.

Exclusion criteria:

- (1) Absence of follow-up information.
- (2) Absence of cytologic information.
- (3) Absence of slides to verify the cytologic pattern in cases without cytology report in the database.
- (4) Unclear or nonconclusive cytologic findings in cases with archived slides but without information in the database.

Based on these criteria, cats were considered as affected by FIP if results of serum and/or effusion protein electrophoresis, AGP serum concentration and cytologic pattern of the effusions were consistent with FIP, and the disease was confirmed postmortem by necropsy and histopathology including positive immunohistochemistry for FCoV as described in a previous study.⁴ Conversely, cats were considered as not affected by FIP if cytology or bacteriology of the effusion was diagnostic for a disease other than FIP, eventually confirmed by necropsy and histology, or if the follow-up revealed rapid improvement of the clinical condition after treatment.

All specimens were submitted to our laboratory for routine diagnostic purposes and were subjected to cell counts by Sysmex, measurement of specific gravity and protein concentration by refractometry (Clinical refractometer Mod. 105; Sper Scientific, Scottsdale, AZ, USA), and cytologic analysis. Necropsies and additional postmortem tests were performed by the routine necropsy services of DIVET. All data were evaluated by 2 ECVCP-certified clinical pathologists unaware of the Δ TNC results.

Sysmex XT-2000iV analysis

According to the standard operating procedures of our laboratory only effusions collected in EDTA tubes and submitted no later than 12–18 hours after sampling are analyzed on the Sysmex XT-2000iV (Sysmex Europe GmbH, Norderstedt, Germany) analyzer to determine TNCC provided by both the DIFF (TNCC-DIFF) and BASO (TNCC-BASO) channels, as well as the

Δ TNC. Specifically, the DIFF channel classifies all nucleated cells based on complexity and nucleic acid content. The BASO channel classifies nucleated cells based on volume and the complexity of cellular residues produced after contact with an acidic reagent that, in human blood, condenses all the nucleated cells except basophils.¹⁴ As effusions include cells other than WBC, the total WBC count and the Δ WBC generated by the instrument were defined as TNCC and DTNC, respectively for the purpose of this study.¹¹

Evaluation of diagnostic sensitivity and specificity

Statistical analysis was performed in an Excel (Microsoft Corp, Redmond, WA, USA) spreadsheet using the Analyse-it software (Analyse-it Software Ltd, Leeds, UK). Results for TNCC-DIFF, the TNCC-BASO, and Δ TNC recorded in cats with and without FIP were compared to each other with a nonparametric *t*-test (Mann–Whitney *U*-test), using the 95% confidence interval (CI) as a measure of uncertainty. To assess the diagnostic accuracy of Δ TNC, the number of true-positive (TP), false-positive (FP), true-negative (TN), and false-negative (FN) results were calculated as follows: TP are the specimens from cats with FIP with a Δ TNC higher than each operating point; TN are the specimens from cats without FIP with a Δ TNC lower than each operating point; FP are the specimens from cats without FIP with a Δ TNC higher than each operating point; and FN are the specimens from cats with FIP with a Δ TNC lower than each operating point.

Using these numbers, sensitivity and specificity were calculated using standard formulas¹⁵ and using the 95% CI as a measure of uncertainty. In addition, the positive and negative likelihood ratio (LR+ and LR–, respectively) were calculated using the formulas: $LR+ = (\text{sensitivity}) / (1 - \text{specificity})$ and $LR- = (1 - \text{sensitivity}) / (\text{specificity})$, respectively.¹⁶

Finally, receiver operating characteristic (ROC) curves were designed by plotting sensitivity vs 1–specificity to determine the discriminating power of Δ TNC to identify cats with FIP.¹⁶ In addition, the optimal cut-off value, corresponding to the operating point closer to the upper left corner of the graph was identified.

Analytic precision and accuracy

Analytic precision and accuracy of Sysmex counts on feline effusions not associated with FIP had already been evaluated in a previous study.¹¹ Specifically, intra-assay coefficient of variation (CV) was 11.5% for TNCC-DIFF and 0.5% for TNCC-BASO, and regression coefficients of specimens read after serial dilutions

were > 0.99 for both TNCC-DIFF and TNCC-BASO. In the same study, a poor repeatability and linearity under dilution of a few specimens from cats with FIP were reported, but no information on the actual repeatability and linearity under dilution of Sysmex readings of TNCC-DIFF and TNCC-BASO of effusions from cats with FIP, or information about precision and accuracy of the Δ TNC were reported.

Therefore, in the current study, repeatability was assessed only on 2 FIP samples with a high Δ TNC and on 2 specimens with a normal Δ TNC in 5 consecutive measurements on one day, and by calculating the CV with the formula: $CV = \text{mean} / \text{SD} \times 100$. To assess linearity under dilution, one specimen each with high and one with normal Δ TNC were serially diluted at 1:1, 1:3, 1:7, and 1:15 (vol/vol) with isotonic saline, resulting in dilutions corresponding to 50%, 25%, 12.5%, and 6.25% of the original effusion, respectively. Specimens were then analyzed on the Sysmex as described above. Linearity was determined by comparing the expected values for each dilution to the values released by the instrument in a linear regression analysis.

Results

Results of the retrospective study population and distribution of cases per group

The retrospective search of the database identified 67 feline effusions originating from cats of different age, sex, and breed that were processed during the study period (June 2009 to June 2013) (Figure 1). Among these, 16 were excluded due to nonconclusive cytologic findings and the lack of follow-up information or postmortem results.

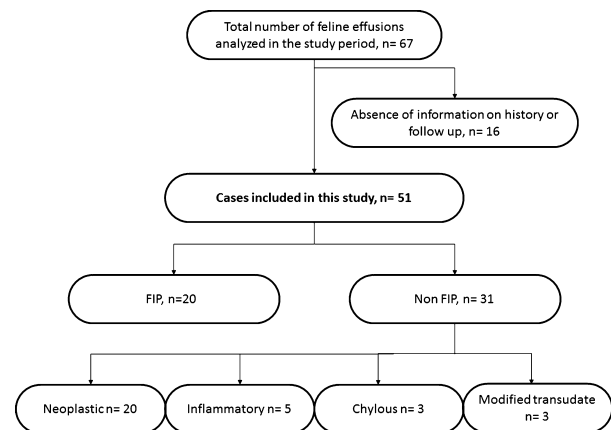


Figure 1. Flow diagram summarizing the inclusion and exclusion criteria for the selection of cases from the database and the final assignment to the study groups.

The remaining 51 effusions were grouped as follows:

Group A, FIP (n = 20). In all these cases except 2, the physico-chemical features and cytology of the effusions were consistent with FIP, showing usually non-degenerate neutrophils, macrophages, lymphocytes and rare plasma cells, and mesothelial cells in a granular proteinaceous background. The 2 cases of FIP with “atypical” findings in the effusion included cat 5 with polyclonal gammopathy and very high serum AGP concentration, but an effusion with a low protein concentration (17 g/L), low specific gravity (1.010), and low cellularity ($0.13 \times 10^9/L$), with rare neutrophils and mesothelial cells in the absence of the proteinaceous background. The pericardial effusion of cat 25 revealed a high number of reactive mesothelial cells, sometimes with evident cytophagia and a weakly proteinaceous background. However in both cases, necropsy revealed the typical subserosal fibrinous lesions (associated with multiple hemorrhages in cat 5), and the diagnosis of FIP was confirmed by histology and the immunohistochemical detection of intralésional FCoV. Necropsy, histology, and immunohistochemistry confirmed FIP in all other 18 cats.

Group B, non-FIP (31 cats). This group included neoplastic effusions ($n = 20$) due to lymphoma ($n = 10$) or epithelial tumors ($n = 8$), diagnosed by cytology of the effusion, and one thymoma and hemangiosarcoma each diagnosed by the detection of unclassified atypical cells in the effusion and by diagnostic imaging, followed, in the case of the hemangiosarcoma, by post-mortem and histologic examination. Furthermore, there were exudates associated with inflammatory conditions ($n = 5$) diagnosed by cytology of the effusion that revealed a prevalent population of neutrophils, in 3 cases associated with positive bacteriology of the effusion, and in 2 cases associated with clinical and laboratory findings consistent with feline cholangiohepatitis. All these cats recovered after appropriate treatments. In addition, there were chylous effusions ($n = 3$) with the typical macroscopic and cytologic appearance¹⁷, and associated with cardiologic abnormalities. Modified transudates ($n = 3$) were in 2 cases associated with intraabdominal tumors confirmed at necropsy, and in one case diagnosed in a cardiopathic cat in which the treatment led to the remission of clinical signs, including the effusion.

Repeatability and linearity under dilution

As shown in Table S1, repeatability of specimens with normal Δ TNC was better for both DIFF and BASO counts as well as for Δ TNC, with CV < 2.56%.

Conversely, CV was higher and extremely variable for the specimens with high Δ TNC, due to a high variability in both BASO and DIFF counts which in turn induced a high variability in Δ TNC.

Linearity under dilution provided excellent results for TNCC-DIFF and TNCC-BASO of the specimens with normal Δ TNC, with correlation coefficients of 0.99 and 1.00, respectively ($P < .001$). Consequently, Δ TNC remained constant over the different dilutions and did not correlate with the values expected after dilution ($r = .81$; $P = .390$) (Figure S1). Conversely, the linearity under dilution of specimens with high Δ TNC was satisfactory only for DIFF-TNCC ($r = .98$; $P = .001$), while DIFF-BASO did not show the expected decreasing counts and basically provided similar results independently of the dilution ($r = .02$; $P = .825$). Consequently, Δ TNC decreased in a linear manner ($r = .98$; $P = .001$) with increasing dilution (Figure S1).

Comparison of TNCC-DIFF, TNCC-BASO, and Δ TNC between cats with and without FIP

The Δ TNC was significantly higher ($P < .001$) in cats with FIP (median: 9.3; min–max: 0.5–36.4) than in non-FIP cats (1.0; 0.5–2.5), and TNCC-BASO and TNCC-DIFF counts were significantly lower ($P < .001$ and $P < .05$, respectively) in cats with FIP (TNCC-BASO = 0.2; 0.0–5.3; TNCC-DIFF = 1.5; 0.1–26.3) than in non-FIP cats (TNCC-BASO = 10.1; 0.0–707.9. TNCC-DIFF = 9.1; 0.1–921.8; Figure 2). Results from these latter cats were characterized by a high inter-individual variability, likely due to the heterogeneity of the diseases responsible for the effusions. All the cats with FIP had a Δ TNC > 3.0, except for the 2 cats which had “atypical” FIP (cat 5 Δ TNC 0.538, cat 25 Δ TNC 1.165). All non-FIP cats had a Δ TNC < 3.0. More specifically, only 2 specimens from all 31 cats without FIP had a Δ TNC > 1.7. These latter 2 cases were a cat with lymphoma with a highly cellular effusion (TNCC-DIFF $25.45 \text{ cells} \times 10^9/L$), and the other specimen was a modified transudate from a cardiopathic cat that was almost acellular (TNCC-DIFF 0.05 cells $\times 10^9/L$, TNCC-BASO 0.02 cells $\times 10^9/L$).

Diagnostic accuracy of Δ TNC

The area under the ROC curve for Δ TNC (Figure 3) was 0.94 (95% CI = 0.84–1.00, $P < .001$ compared with the line of no discrimination). The best cutoff for Δ TNC determined by the ROC curve analysis was 1.7. At this value, sensitivity was 90.0% (95% CI = 68.3–98.8%), specificity was 93.5% (95%

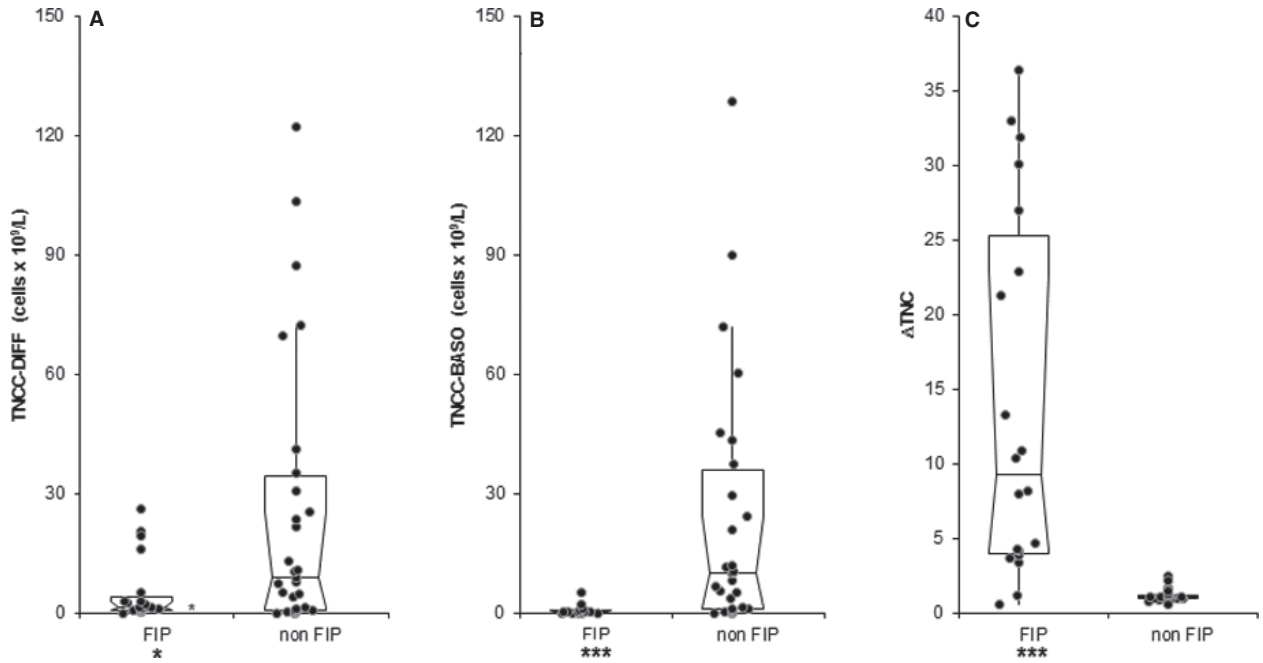


Figure 2. Values of TNCC-DIFF (A), TNCC-BASO (B), and the ratio between total nucleated cell counts in the DIFF and BASO channel of the laser counter Sysmex XT-2000iV, reported as Δ WBC by the instrument but termed Δ TNC for the purpose of this study (C) recorded in cats with FIP and in cats with diseases other than FIP (non-FIP). The boxes indicate the I–III interquartile range (IQR), the horizontal line indicates the median, whiskers extend to within the I quartile minus 1.5*IQR or to within the III quartile plus 1.5*IQR. Dots indicate the values recorded in this study. The TNCC-DIFF and the TNCC-BASO graphs do not include the result of a neoplastic non-FIP specimen that had extremely high TNCC-DIFF and TNCC-BASO counts (921.8 and 707.9 cells \times $10^9/L$). The black bolded asterisks reported below the X-axis indicate a significant difference between groups (* P < .05; *** P < .001).

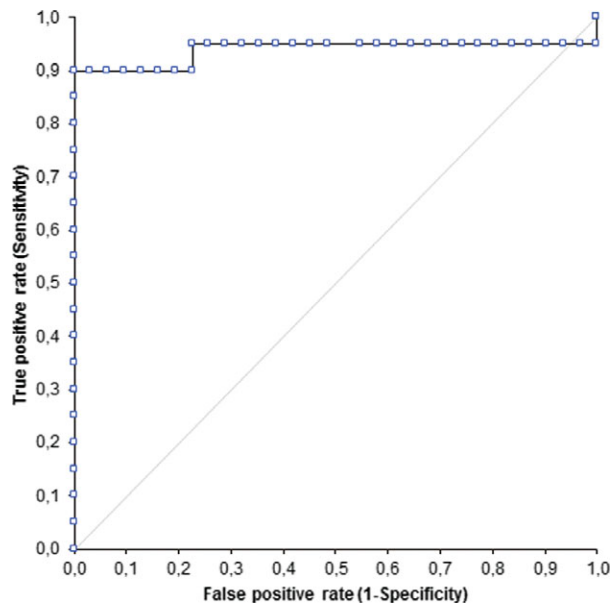


Figure 3. Receiver operating characteristic (ROC) curves of the ratio between total nucleated cells in the DIFF and BASO channel of the laser counter Sysmex XT-2000iV, reported as Δ WBC by the instrument but termed Δ TNC for the purpose of this study, for the diagnosis of FIP. The gray line indicates the line of no discrimination.

CI = 78.6–99.2%), LR+ was 13.9 (95% CI = 4.6–86.3), and LR– was 0.11 (95% CI = 0.0–0.3). When using a cutoff of Δ TNC = 2.5, specificity increased to 100%.

Discussion

The diagnosis of FIP should be based on a combination of clinical and laboratory findings.² The analysis of effusions is useful to support a clinical diagnosis of FIP or, conversely, a different disease and rule out FIP from the list of differential diagnoses.^{1,5,18} FIP, in contrast to other diseases, is characterized by protein-rich effusions^{17,18} containing a large amount of globulins, particularly γ -globulins^{7,19} and fibrinogen.⁵ It is the latter 2 that react with acidic solution in the Rivalta’s test and visibly clot in the tube. Recently, the Rivalta’s test has been found to be highly diagnostic for FIP, although, as any other test, its specificity and sensitivity are not 100%.⁹ In the present study, it was investigated whether cell counts performed in the laser-based Sysmex XT-2000iV that has the so-called BASO channel in which cells are counted after precipitation in an

acidic reagent provide diagnostic information similar to the Rivalta's test. This was suggested by a previous study that, however, included only a few specimens of effusions from cats with FIP.¹¹ In our study, we had the opportunity to investigate a larger number of cats with or without FIP. Very stringent inclusion criteria for the FIP group granted for a very well-defined positive study population. Unfortunately, the "non-FIP" group was composed largely of neoplastic effusions that were not as challenging for a differential diagnosis from FIP, as the 2 conditions may be easily differentiated by cytology. Therefore, a possible limitation of this study is the low number of nonneoplastic effusions that in routine practice may benefit from an additional test to distinguish FIP from other types of inflammatory or reactive effusions. However, also in the previous feline effusions study¹¹, all the specimens from cats with inflammatory effusions other than FIP had a $\Delta\text{TNC} < 1$, confirming that a $\Delta\text{TNC} > 1$ has a high diagnostic accuracy for FIP.

The results of the current study confirmed that the instrumental analysis with the Sysmex XT-2000iV may represent an additional reliable method for confirmation of FIP in the analysis of effusions. Specifically, as cells are entrapped in clots formed by fibrinogen precipitation in the BASO channel, cell counts in the BASO channel (BASO-TNCC) are usually lower than those of the DIFF channel. This mechanism explains why the ΔTNC (in the Sysmex referred to as ΔWBC) increases in such specimens. Based on our results, ΔTNC has a high diagnostic accuracy for FIP, which was supported by sensitivity and specificity $> 90\%$ and the ROC curve. Specifically, with a positive likelihood ratio close to 14, a $\Delta\text{TNC} > 1.7$ makes it 14 times more likely that an effusion originates from a cat with FIP rather than a different disease. Conversely, a negative likelihood ratio of 0.11 suggests that with a $\Delta\text{TNC} < 1.7$, the probability that the effusion comes from a cat with FIP is about one-tenth of the probability that the effusion comes from a cat with a different disease.

The specificity is 100% with $\Delta\text{TNC} > 2.5$, a value that was ultimately found in all FIP effusions except the 2 atypical cases. Specifically, the effusion in one cat with hypoalbuminemia and a hemorrhagic syndrome was classified as a transudate.²⁰ All these changes are consistent with liver failure that may induce also hypofibrinogenemia, likely preventing clotting in the BASO reagent. In the other case, the cytologic pattern of the effusion was complicated by the presence of "atypical" mesothelial cells that are usually less abundant in FIP effusions than neutrophils and lymphocytes. However, this may represent a feature typical for pericardial effu-

sions, often leading to a misdiagnosis of neoplasia.¹⁷ Therefore, in both cases, the false-negative results of ΔTNC may be related to atypical features of the effusion rather than the low analytic sensitivity. As regards specificity, only 2 false-positive results were found; one lymphoma case, which in people sometimes provides positive Rivalta's test results¹⁰, possibly due to the presence of fibrinogen associated with an inflammatory reaction against the tumor itself, and one case with a poorly cellular fluid, in which the high ΔTNC was clearly a mathematical artifact due to analytical sensitivity of the instrument. However, both these cases do not represent a diagnostic challenge in routine practice as FIP may be easily excluded if additional investigations such as cytology of effusions are added to the diagnostic workup. Independently on these few cases, the analysis of effusions with the Sysmex-XT2000iV counter evidenced a sensitivity and a specificity comparable to or even higher than that previously reported for the Rivalta's test⁹, likely because the mechanisms are very similar. The Rivalta's test is rapid, cheap, and accurate, but it may be limited due to some preanalytic or analytic factors. For example, the test may be inaccurate due to inappropriate techniques or intrinsic factors of the reagents such as concentration of acetic acid, different temperatures of the effusion, and the acetic acid solution. Moreover, the reaction may be negative when pH increased to 4.6 or higher.^{8,10} Additionally, the Rivalta's test provides semi-quantitative results (negative, weakly or strongly positive) and does not allow grading the severity of the change. Finally, the evaluation of the test is subjective and no information about inter-observer variability is currently available. Conversely, the analysis with the Sysmex-XT2000iV counter is more standardized in terms of reagents, although the repeatability study demonstrated that, limited to FIP effusions, it may suffer from a poor precision which however did not affect the interpretation of the results, as ΔTNC was always > 1.0 . Moreover, the test is rapid and, in one single measurement, provides information on the presence of precipitable proteins, and a provisional information on the cell types based on the scattergram¹¹ in addition to the cell count. However, it must be stressed that the linearity under dilution test performed in this study demonstrated that the more accurate cell count provided by the instrument is the DIFF-TNCC that is not affected by the entrapment of cells in the clots formed after contact with the BASO reagent. Therefore, in routine practice, it is not recommended to use the default WBC counts that are generated by the BASO channel. Conversely, when FIP is clinically suspected, it may be recommended to directly check the results of

the DIFF-TNCC and the Δ TNC that are reported in the Service screenshot of the software. Moreover, in future studies, it may be interesting to assess whether other laser-based instruments such as those of the ADVIA series that use a similar analytic principle to count basophils in peripheral blood¹⁴ provide the same interesting results on FIP effusions.

Conclusion

In conclusion, this study evidenced a very high diagnostic accuracy of the Sysmex-generated Δ TNC for the diagnosis of FIP. This depends on the formation of clots in the BASO reagent that entrap the cells, similarly to what occurs in the Rivalta's test that has also been reported to have a high diagnostic accuracy for FIP. This reaction leads to a low BASO-TNCC even when DIFF-TNCC counts are high. Therefore, in routine practice, it is not recommended to use the default TNCC counts generated by the BASO channel, but to directly use the DIFF-TNCC and especially the Δ TNC, particularly when FIP is suspected. In these cases, a Δ TNC > 1.7 is highly suggestive of FIP, and a Δ TNC > 3.4 may be considered diagnostic for FIP.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Linearity under dilution (LUD) recorded in serially diluted effusion specimens from a cat with lymphoma (A, B, C) and in a cat with FIP (D, E, F).

Table S1. Results regarding repeatability recorded in 2 cats with high Δ TNC associated with FIP and in 2 cats with normal Δ TNC.