ESVCP Mystery Case 2021

CONTRIBUTOR NAME*	Maud Guerlin
CONTRIBUTOR EMAIL*	maud.guerlin@envt.fr
COAUTHORS	Fanny Granat, Catherine Trumel
COMPANY OR UNIVERSITY	Toulouse University, ENVT

* Corresponding contributor

SIGNALMENT:

A 8 month-old intact male Staffordshire Bull Terrier dog.

HISTORY AND CLINICAL FINDINGS:

The dog was presented with a 24 hours-history of lethargy, anorexia, vomiting and pigmenturia (probably hemoglobinuria).

On physical examination, the dog was dehydrated and severely lethargic. He had pale and tacky mucous membranes, increased breath sounds and a prolonged capillary refill time.

No abnormality was noted through a complete (abdominal and thoracic) radiographic and ultrasonographic investigation.

Complete blood cell count (CBC), blood film, biochemical and urine analysis were also performed. Results are in tables 1, 2 and 3.

Table 1: Hematology results obtained with the Sysmex XN-V® (Sysmex)

Analytes	Data	Reference Interval	Alarm
HGB (g/dL)	10	12.4-19.2	
RBC (.10^{12}/L)	3.4	5.2-7.9	
HCT (L/L)	0.29	0.35-0.52	
MCV (fL)	75.9	60.0-71.0	
MCH (pg)	25.9	21.9-26.3	
MCHC (g/dL)	34.1	34.4-38.1	
RDW-SD (fL)	36.2	31.1-38.9	
RDW-CV (%)	13.2	13.2-19.1	
PLT-I (.10^{9}/L)	174	64-613	
PLT-O (.10^{9}/L)	234	108-562	
PLT-F (.10^{9}/L)	204	-	

WBC-WDF (.10^9/L)	2.41	5.60-20.40	*
WBC-WNR (.10^9/L)	22.31		« *Difference between WNR and WDF. Check the results*. »
Neutrophils (.10^9/L)	3.02	2.90-13.60	*
Lymphocytes (.10^9/L)	9.67	1.10-5.30	*
Monocytes (.10^9/L)	0.02	0.40-1.60	*
Eosinophils (.10^9/L)	5.45	< 3.10	*
Reticulocytes (10^6/µL)	76.8	19.4-150.1	« *RET Abn Scattergram* »
Reticulocytes (%)	1.99	0.30-2.37	
SNAP test 4 Dx Plus® (Idexx) (*Dirofilaria immitis, Ehrlichia canis, Ehrlichia ewingii, Anaplasma phagocytophilum, Anaplasma platys,* and *Borrelia burgdorferi*)	Negative	Negative	

*: indicates low reliability

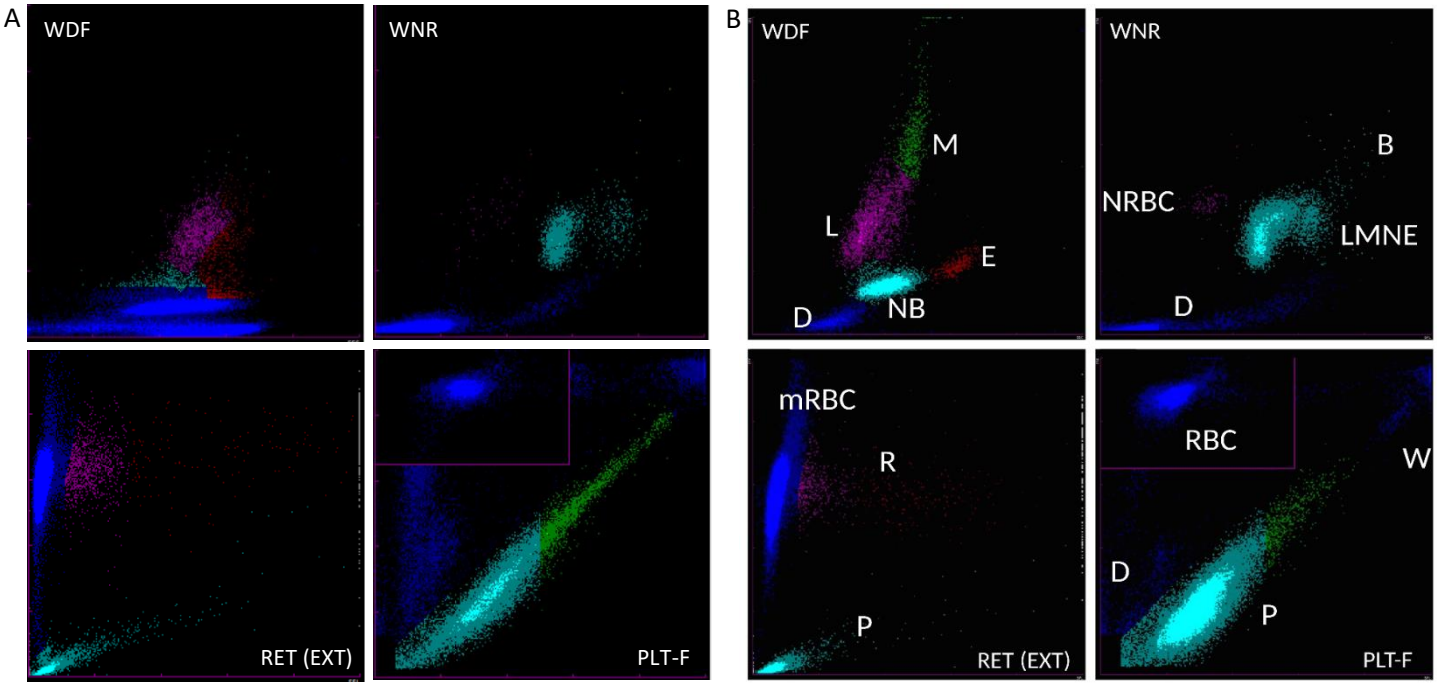
Table 2: Biochemistry results (Vitros®, Ortho Clinical Diagnostics Inc.)

Analytes	Data	Reference Interval
Color of the plasma	Dark orange to red	Clear
Glucose (mmol/L)	7.5	3.7-8.2
BUN	7.3	1.6-10.9
Creatinine (µmol/L)	41.5	44.0-133.0
AST (U/L)	143	1-37
ALT (U/L)	99	3-50
PAL (U/L)	115	20-155
GGT (U/L)	7	5-25
CK (U/L)	241	25-467
Total Bilirubin (µmol/L)	19.6	1.7-12.0
Total Protein (g/L)	58	48-66
Albumin (g/L)	31	23-39

Table 3: Non verified and partial urinalysis results (performed by a student at the emergency unit)

Analytes	Data	Reference Interval
Source	Unknown	-
Color	Dark red	Clear yellow
DU	> 1.060	1.015-1.045
Sediment	Red blood cells : non quantified Bilirubin crystals +++	< 5 cells / 40 PF
Dipstick	Not performed	-

Figure 1: Sysmex XN-V® cytograms from peripheral blood sample.



A: 8 month-old Staffordshire Bull Terrier dog. B: Healthy dog for cell identification.

WBC differential scattergram (WDF); WBC count scattergram (WNR); Reticulocyte extended scattergram (RET(EXT)) and platelet scattergram with optic-fluorescent analysis (PLT-F) ; B, basophils; D, debris; E, eosinophils; L, lymphocytes; M, monocytes; mRBC, mature RBC; N, neutrophils; NRBC, nucleated red blood cells; P, platelets; R, reticulocytes; RBC, red blood cells ; W, white blood cells.

Figure 2: Photomicrographs of peripheral blood smear of the dog (modified May-Grünwald Giemsa stain, A: x 200; B-C: x 1000, oil). Black arrow: Heinz Bodies; red arrow: ghost cells; white arrow: eccentrocytes; black arrowhead: free Heinz Bodies.

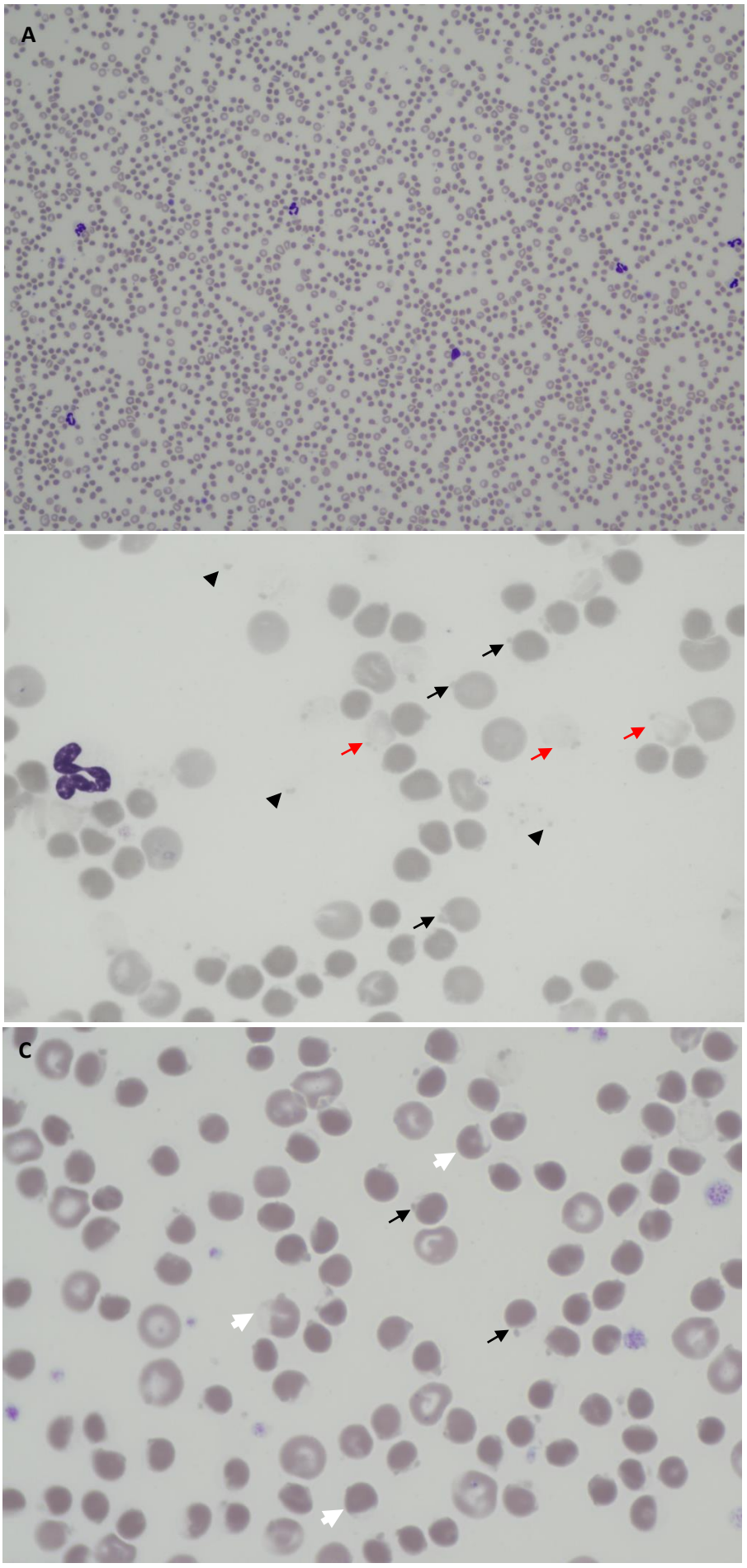


Figure 3: Photomicrograph of peripheral blood smear of the dog, Testsimplets® (Waldeck), x 1000, oil. Red arrow: Heinz Bodies.

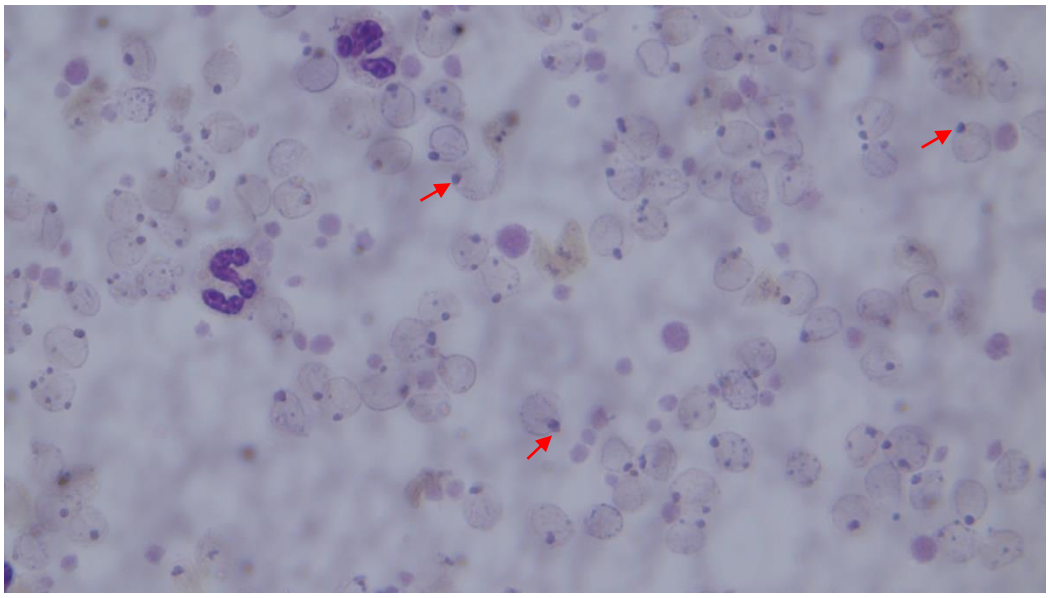


Figure 4: Macroscopic appearance of the heparinized plasma

QUESTIONS:

Question 1: Based on the blood smear evaluation, what is the most likely hypothesis of anemia in this dog?

Question 2: Can you explain the discrepancy between the WBC scattergrams and their unusual aspect?

CLINICAL CHANGES DESCRIPTION:

Hematology results obtained with the Sysmex XN-V® analyzer showed a moderate macrocytic hypochromic regenerative anemia, a marked discrepancy between the two quantitative results from the WBC channel (WBC-WNR = 18.19.10^9/L; WBC-WDF = 2.41.10^9/L) and a platelet count within the reference interval with a mild discrepancy between the three PLT channels (impedance (PLT-I), optical (PLT-O) and fluorescence (PLT-F)) (Table 1).

Abnormal dot plots were observed on WBC-WNR, WBC-WDF, RET-EXT and PLT-F scattergrams. Alarm messages were reported for the red blood cells population ("*RET Abn Scattergram*") and leukocytes population ("*Difference between WNR and WDF*"). Flags were noted for the WBC-WNR, WBC-WDF and the differential WBC ("*") (Table 1).
The WBC-WDF scattergram was characterized by a down shift of the dot plots towards the x-axis mainly associated with two main large basal dot plots and no clear distinction between the different WBC subtypes. The WBC-WNR scattergram was characterized by a large debris dot plot near the x-axis and a slightly decreased WBC dot plot compared to the healthy dog. On the RET-EXT and the PLT-F scattergrams a subpopulation was noted, falling between the red blood cells and the platelets dot plots along the y-axis. (Figure 1)

On the blood smear, the semi-quantitative evaluation of the WBC count was normal and seems consistent with the WBC-WNR channel count. Moreover, contrary to the WBC differential given by the WBC-WDF channel, the WBC population was mainly composed of neutrophils (85.0% manual *vs.* 16.5% automated) and in a lesser extent of monocytes (9.0% manual *vs*. 0.1% automated) and lymphocytes (6.0% manual *vs*. 53.2% automated).
The red blood cells population looked reduced in number. Marked anisocytosis, mild polychromasia, some Howell-Jolly bodies, few basophilic stippling and nucleated red blood cells were consistent with a regenerative process as the reticulocyte count was increased. The most significant abnormalities were a high amount of Heinz bodies (on almost 100% of the red blood cells and sometimes free on the background), many ghost cells and numerous eccentrocytes (Figure 2).
No significant change was noted for the platelets population at microscopic evaluation.
A Testsimplets® prestained slide (containing methylene blue-N and cresyl violet acetate) confirmed the presence of numerous Heinz bodies (Figure 3).

The macroscopic appearance of the heparinized plasma was abnormal with a dark orange to red color (Figure 4) suggestive of mild intravascular hemolysis. The plasma biochemistry significant changes were a moderate increase of ALT and AST activities and a marked increase of total bilirubinemia. On urinalysis, a suspicion of marked pigmenturia (probably at least hemoglobinuria and maybe hematuria) was noticed but urinalysis was incomplete and not verified (Table 3).

Some vector borne diseases were excluded as possible infectious causes of regenerative anemia based on the negative Snap test 4Dx Plus (Idexx, USA) results (Table 1).

INTERPRETATION/DIAGNOSIS:

Based on clinico-pathological findings, a diagnosis of hemolytic anemia was made, and the examination of the blood smear was highly suggestive of a haemolytic anemia secondary to a severe oxidative injury with formation of Heinz bodies and eccentrocytes, and most probably due to an undetermined intoxication (for example: acetaminophen, onion, garlic, zinc, chlorate…).

CLINICAL OUTCOME AND FOLLOW-UP:

Due to financial difficulty the dog could not be hospitalized and received only an antioxydant drug (acetylcysteine). Unfortunately the condition of the animal deteriorated and it died as a result of a cardiovascular arrest a few days later.

ANSWERS TO QUESTIONS:

Question 1: Based on the blood smear evaluation, what is the most likely hypothesis of anemia in this dog?

On the blood smear evaluation, the red blood cell population looked reduced in number. Marked anisocytosis, mild polychromasia, some Howell-Jolly bodies, few basophilic stippling and nucleated red blood cells were consistent with a regenerative process confirmed by an increase of the reticulocyte count. A high amount of Heinz bodies (on almost 100% of the red blood cells and sometimes free on the background), many ghost cells and numerous eccentrocytes were observed. This was highly suggestive of an haemolytic anemia secondary to a severe oxidative injury with formation of Heinz bodies and eccentrocytes. Poisoning was highly suspected in this case (for example: *Alliacaea*, acetaminophen, zinc, naphthalene, vitamin K, skunk musk, …), congenital diseases have also been suspected whereas other causes such as spontaneous diseases remained less likely given the age of the patient (i.e diabetes mellitus, lymphoma, …).

Question 2: Can you explain the discrepancy between the WBC scattergrams and their unusual aspect?

The main modifications of the CBC were the abnormal scattergrams and the marked discrepancy between the quantitative results from the WBC-WNR and WBC-WDF channels. The WBC-WDF scattergram was characterized by an abnormally low fluorescent signal, a down shift of the dot plots towards the x-axis with two main large basal dot plots and no clear distinction between the different leukocytes subtypes. The WBC-WNR scattergram was characterized by a slightly decreased WBC dot plot compared to the healthy dog. Both were characterized by enlarged dot plots in the debris area, especially the WDF channel. These abnormal scattergrams result in a marked discrepancy between WBC counts from the WDF and WDR channels.
In this case of Heinz body hemolytic anemia, the red blood cell lysis in WBC chambers with the release of abnormal hemoglobin, such as denatured globin portion and/or methemoglobin, is suspected to interfere with the leukocytes fluorescent staining. As reported in human cases of congenital hemoglobin variant and methemoglobinemia, the fluorescent dye of the WBC channels (polymethine) could combine with greater affinity to the abnormal hemoglobin than to leukocytes nucleic acids and the abnormal hemoglobin could reduce the permeability of leukocytes to polymethine. In our case, we suspect denaturated globin portion of hemoglobin (Heinz bodies) and/or methemoglobin to have similar affinity for polymethine than reported with instable hemoglobin in human literature.
As reported in human medicine in cases of hemoglobin variant, we can also hypothesize that the unstable hemoglobin (Heinz Bodies and/or methemoglobin) could explain an incomplete lysis of red blood cells and the plotting of unlysed erythrocytes on the WBC scattergrams with enlarged dot plots in the debris area.

DISCUSSION:

We report a case of severe oxydative injury with secondary haemolytic anemia in an 8 month-old intact male Staffordshire Bull Terrier dog. The blood smear evaluation revealed numerous Heinz bodies and eccentrocytes. Heinz bodies are inclusions that form within the red blood cells secondary to oxidative denaturation of the globin portion of hemoglobin with clumping on the inner surface of the red blood cells. Eccentrocytes are formed as a result of direct oxidative damage to the red blood cell membrane with adhesion of the opposing faces of the membrane. Oxydative damages then lead to increased red blood cell fragility and premature phagocytosis (1). The color of the plasma was moderately orange to red, suggestive of a moderate intravascular hemolysis mechanism. Even if the final etiologic diagnosis remains unknown, poisoning was highly suspected in this case (for example: *Alliacaea*, acetaminophen, zinc (2), naphthalene (3), vitamin K, skunk musk (4), …), congenital diseases have also been suspected whereas other causes such as spontaneous diseases remained less likely given the age of the patient (i.e diabetes mellitus, lymphoma, …) (1, 5).

The main modifications of the CBC were the abnormal scattergrams obtained with the Sysmex XN-V® analyzer and the marked discrepancy between the quantitative results from the WBC-WNR and WBC-WDF channels (Figure 1). The WBC-WDF scattergram was characterized by a down shift of the dot plots towards the x-axis with two main large basal dot plots and no clear distinction between the different leukocytes subtypes. Regarding the WBC population, different alarms were given by the Sysmex XN-V®. The device displayed an alarm message "*Difference between WNR and WDF. Check the results*.", but it didn't appear on the final results sheet and hence the importance of paying close attention to scattergrams and WBC counts on the results sheet. Asterisks were also noticed on the results sheet for the WBC count, the complete differential count and NRBC count, indicating low reliability of these data. On the blood smear evaluation, the leukocytes quantitative assessment seemed normal and most consistent with the WNR count. A manual leukocytes estimation was not performed to control the Sysmex XN-V® measurement and a device misjudgment is possible as the WNR scattergram slightly differs from the healthy dog and because of the mentionned low reliability of the results.
This discrepancy between the WBC channels and these abnormal dot plots on the WBC differential scattergram have been previously reported in human medicine with the Sysmex XN and XE-Series analyzers secondary to congenital hemoglobin variants and more recently in acquired conditions such as methemoglobinemia secondary to poisoning (6-11).

In the Sysmex XN-Series analyzers, the total WBC count is provided by the WBC-WNR channel while the WBC differential is provided by the WBC-WDF channel, and both are performed by flow cytometry (FSC: forward scatter light or SSC: side scatter light) and polymethine fluorescence dying (SFL : side fluorescent light). In both WNR and WDF chambers, the reagents induce hemolysis and increase the leukocytes membrane permeability to allow the fluorescent dye to combine with nucleic acids in permeabilized cells. Then, the intensity of fluorescence reflects the nucleic acid content of the cells. (12)

Spurious cell counts and scattergrams have already been described in a feline Heinz body hemolytic anemia with the Sysmex XT-Series (13), but to the author's knowledge this is the first canine report for the Sysmex XN-Series. One of the typical modification noticed in the human literature (6-11), as in our case, is the abnormally low fluorescent signal in the WDF channel. Even if the mechanism is not fully understood, it is suspected that red blood cell lysis, and consequent release of unstable hemoglobin (congenital hemoglobin variant or methemoglobinemia), could interfere with the leukocytes staining. First, the polymethine could

combine with greater affinity to the abnormal hemoglobin than to leukocytes nucleic acids. Secondly, the abnormal hemoglobin could reduce the permeability of leukocytes to polymethine (6-11). In our case, methemoglobinemia has not been quantified but Heinz bodies were present. It can be hypothesized that methemoglobin was present in small quantity, even if cyanotic-appearing mucous membranes were not noticed, and/or that denaturation of the globin portion of hemoglobin could have similar affinity for polymethine than reported with instable hemoglobin. Nevertheless, a spot test, a blood gas analysis or a spectrophotometric measure would have allowed to investigate the possibility of methemoglobinemia and thus its eventual contribution to the observed abnormalities.

Another key modification is the enlarged dot plots in the debris area of the WBC channels (Figure 1). As reported in human medicine in cases of hemoglobin variant, we can also hypothesize that the unstable hemoglobin (Heinz Bodies and/or methemoglobin) could explain an incomplete lysis of red blood cells and the plotting of unlysed erythrocytes on the WDF scattergram (6-11). As previously reported in two cats with Heinz body hemolytic anemia (14) as well as in human literature (6-11), this WBC scattergram modification is not observed with analyzers using myeloperoxydase channel such as the the Advia 2120 (Siemens Medical Solution USA Inc.). Moreover, in our case as in human literature, the WNR channel seems less affected than the WDF. This means that it depends on the analyzer and the reagents used (concentration and properties such as acidity, osmolarity, ...). In the WNR channel of the XN-series, the concentration of polymethine is higher than in the WDF channel, a higher efficacy of the lysing reagent is so suspected, permitting quicker red blood cell lysing and haemoglobin variant elimination before the action of the fluorescent dye (11).

On the RET-EXT and the PLT-F scattergrams, there is a quite good distinction between the different dot plots. However, the device displayed an alarm message ("*RET abnormal scattergram*") and a subpopulation was observed falling between the red blood cells and the platelets dot plots along the y-axis, with a consequent possible confusion between these two dot plots on both scattergrams (Figure 1). This specific area below the RBC on the RET-EXT scattergram is used for identification of fragmented RBC. In RBC fragments as in intact RBC the fluorescence signal (SFL) is low due to the absence of nucleic acids. The MCV of RBC fragments is lower than intact RBC, with a consequent lower FSC (15, 16).

Altered PLT and RBC scattergrams have previously been reported in human and veterinary medicine with various analyzers due to RBC fragmentation and oxydative damage (13, 15, 17, 18). Fragmented or microcytic RBC have cell volume and fluorescent index similar to platelets and thus can not be distinguished with the Sysmex XT-2000iV (15). However, in human medicine the newly developed PLT-F channel in the XN-Series has been reported to be a more reliable platelet count system than PLT-I and PLT-O channels, even in abnormal samples containing fragmented erythrocytes, thanks to its accurate and specific fluorescent dye (oxazine) (19, 20). The PLT-F reagents strongly stain intraplatelet organelles, such as ribosomes and mitochondria, and only faintly stain the plasma membrane of both platelets and erythrocytes. This method is also highly correlated with the immunological reference method in human medicine but have never been validated in dogs and cats (19). In our case, it can be hypothesized that abnormal RBC (Heinz bodies, free Heinz Bodies, ghost cells and eccentrocytes) explain the abnormal dot plot along the y-axis between the intact red blood cells and the platelet dot plots. For the platelet count, it is quite challenging to know which count is the most adequate between PLT-O and PLT-F as the latest technology has never been validated in dogs. But the manual platelet count was estimated to at least 252.10^9/L, and so quite consistent with the PLT-O and PLT-F counts. However, few platelets aggregates were present on the blood smear and so the exact platelet count remains unknown.

No manual reticulocyte count was performed. But as the reticulocytes dot plot seems normal on the RET-EXT scattergram, we can be quite confident on the analyzer reticulocyte count.

To conclude, spurious WDF scattergrams and discrepancy between WBC counts from the WDF and WDR channels can be observed in case of Heinz body hemolytic anemia with the Sysmex XN-V® at least in dog. Red blood cell lysis with release of abnormal hemoglobin, such as denatured globin portion and/or methemoglobin, is suspected to interfere with the leukocytes fluorescent staining, although this phenomenon depends on the analyzer and the reagents used. The observation of this abnormal-WDF scattergram should lead to suspect red blood cell oxydative injury or, as reported in human medicine, a congenital hemoglobinopathy due to an unstable hemoglobin variant.

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