Marked pseudoreticulocytosis in a leukemic dog

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Signalment:

A 14 years-old, male castrated, 5 Kg, Coton de Tular dog.

Clinical findings:

The dog was presented to a private veterinary practice for clarifying a gastro-intestinal problem. Referred clinical signs were anorexia, five episodes of vomiting, and weakness since one week. Moreover the night before consultation the dog showed an episode of nocturia. On the clinical evaluation, the dog was bright and alert. Auscultation of the heart and lungs was unremarkable. At abdominal palpation the animal seemed mildly painful; spleno- and hepatomegaly were also appreciated.

Diagnostic procedures:

Initial complete blood cell count (CBC), blood smear and biochemical analysis were carried out by the private veterinarian. On the basis of blood results a moderate regenerative anemia was present. Total number of white blood cells was severely increased and not measurable by the cell counter. However a first blood smear revealed extreme lymphocytosis with atypical cells. Biochemistry analysis was unremarkable. Survey thoracic did not reveal any morphological alteration of the observed structures. Abdominal ultrasound revealed mild increase in splenic and liver volumes without parenchymal focal lesions. On the basis of the first tests, high suspicion of a neoplastic hematologic disorder was advanced by the private veterinarian that subsequently sent blood samples of the dog to the diagnostic laboratory of the University of Milan for subsequent diagnostic tests because of the high suspicion of hematologic neoplastic disorder. Immunophenotyping by flow cytometry (FC) was required.

Description and Diagnosis:

CBC and blood smears were performed at the diagnostic laboratory of the University of Milan. CBC was performed with a laser automated analyzer (Sysmex XT 2000 iV®). A moderate normocytic and normochromic anemia was identified, as shown in Figure 1A. Interestingly, red blood cells counted by optic (RBC-O) resulted higher than the one using electrical impedance (RBC-I), as shown in Figure 1A. Manual PCV was 18% and resulted lower compared to the one obtained by the automated analyzer (22.9%). Abnormal dot plots were observed for DIFF, WBC/BASO, RET and PLT-O scattergrams and two flags were reported by the analyzer: “PLT Abn Distribution” and “WBC Abn Scattergram”. Leukocyte count was repeated after 1:2 dilution in saline solution. After correction for dilution factor, leukocyte counts resulted to be 1’562’680 cells/µL. The reticulocyte percentage was 35.29% and IRF (immature reticulocyte fraction) was 98.2%, as shown in Figure 1B. Medium and high-fluorescence reticulocytes were 36.7% and 61.5%, respectively. The RET-EXT showed a gap between normal RBC and high fluorescence reticulocytes. A large discrepancy was detected between the automated reticulocyte counts and the mild polichromasia present in the blood smear. Manual reticulocytes count was <1% and resulted lower compared to the results obtained by Sysmex XT-2000iV®.

Figure 1A: RBC index (Sysmex XT-2000iV®)

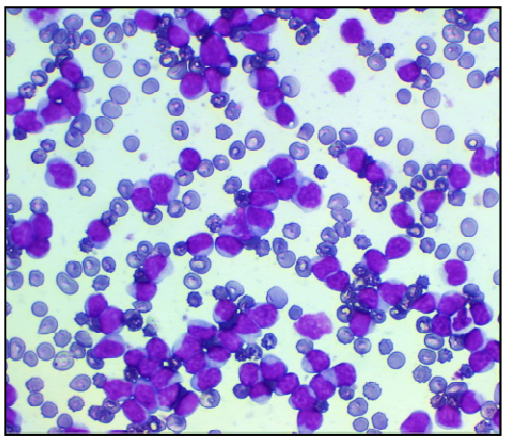
Item	Data		Unit
RBC	3.22	-	10^6/uL
RBC-O	4.40		10^6/uL
HGB	7.9	-	g/dL
HCT	22.9	-	%
MCV	71.1		fL
MCH	24.5		pg
MCHC	34.5		g/dL

Figure 1B: Reticulocytes index

RET#	1.1363	+	10^6/uL
RET%	35.29	@	%
IRF	98.2		%
LFR	1.8		%
MFR	36.7		%
HFR	61.5		%

Blood smear examination showed a prevalent population (99%) of medium sized mononuclear cells (nucleus about 2 rbc in diameter) with small amount of grey-blue cytoplasm, round to indented nucleus with smooth to irregularly clumped chromatin and inconspicuous nucleoli. Mitosis were absent. These cells were highly suggestive of mature lymphocytes.

Figure 2: Smear of blood from the dogs, May-Grünwald Giemsa, 10X

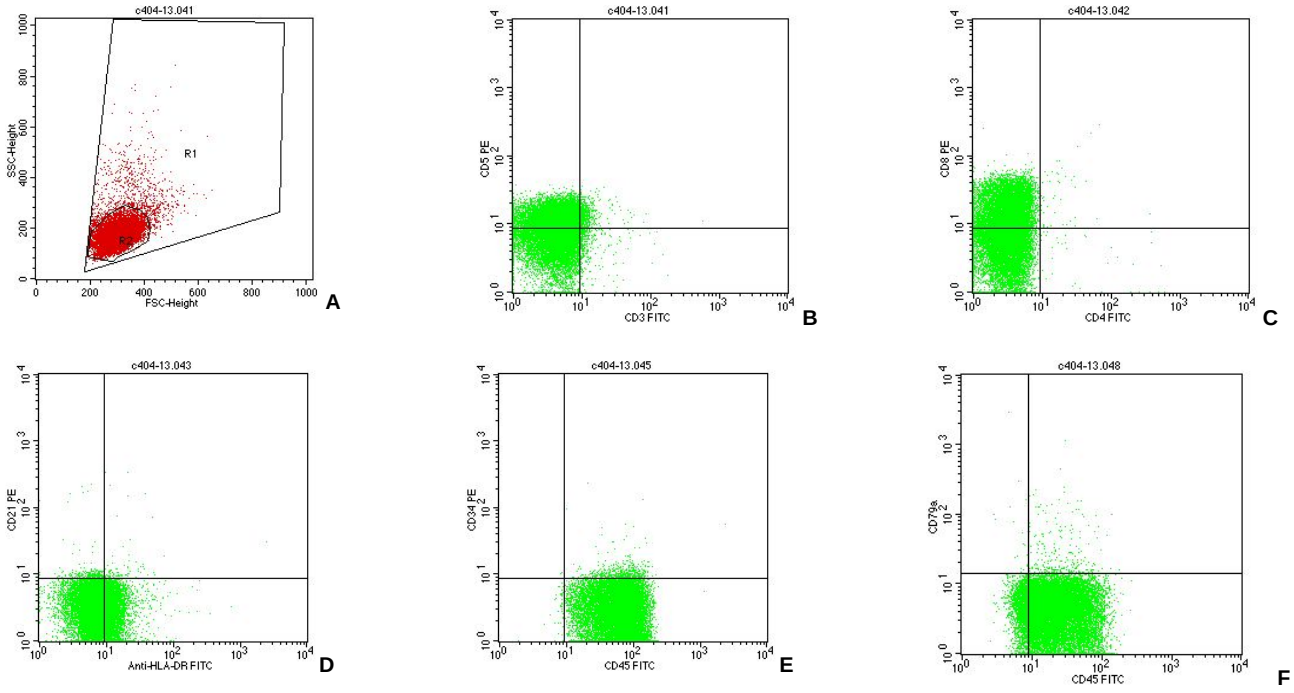


Immunophenotyping of neoplastic cells was performed by flow cytometry. Detected percentages are shown in Table 1 and Figures 3A-F

Table 1:

Marker	Detected cells	Blood
CD45	Pan-leukocyte	100,0%
CD44	Pan-leukocyte	99,0%
CD3	Pan-T lymphocyte	3,5%
CD5	Pan-T lymphocyte	36,6%
CD4	T-helper lymphocyte	1,2%
CD8	T-cytotoxic/ suppressor	38,4%
MHC II	T lymphocyte and monocyte	18,6%
CD21	B-lymphocyte (mature)	0,3%
CD79a	Pan-B lymphocyte marker	<1%
CD117	Hematopoietic precursors	6,7%
CD34	Hematopoietic precursors	3,4%

Figures 3A-F: Specific scattergrams of the blood samples analyzed by flow cytometry



Hematological results and blood smear examination suggested a diagnosis of mature lymphoid cells neoplasia (chronic lymphocytic leukemia -CLL). Immunophenotyping by flow cytometry sustained the final diagnosis of leukemia of T phenotype (T-CLL) in possible progression/transformation.

Treatment and follow up:

After presentation, the dog was treated with chlorambucil (0,2 mg/kg die) and prednisolone (30 mg/m^2 die). In addition, vincristine (0,75 mg/m^2) and cyclophosphamide (250 mg/m^2) were administered. After the second blood check, only chlorambucil was administered. At the last control the dog showed neurological symptoms with head tilt and few episodes of epilepsy. The dog is now receiving a rescue protocol (fludarabine 20mg/m^2 iv). RBC and WBC counts during follow-up are indicated in Figure 4. Specifications of the hematological findings are shown in Table 2.

Figure 4: Treatment and hematological findings during first presentation and follow-up

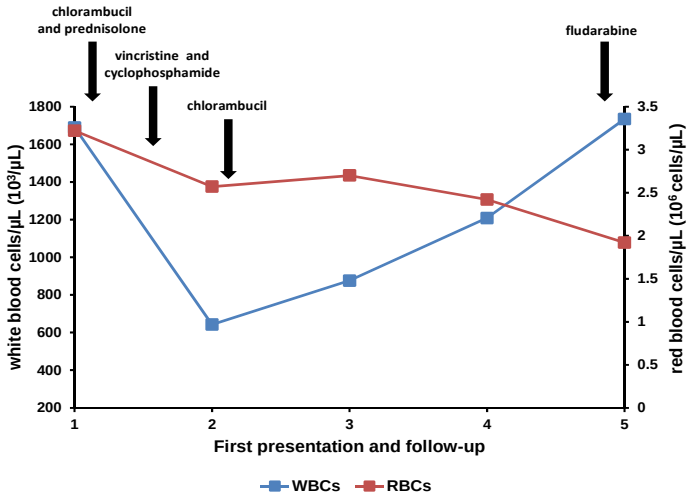


Table 2: Summary table of hematological parameters of the first case presentation (line 1) and subsequent follow-up (lines 2-5)

	Case and follow up	RBC (10^6 cells/µL)	HTC (%)	MCV (fL)	MCHC (g/dL)	WBC (10^3 cells/µL)
1	14.03.13	3.22	22.9	71.1	34.5	1’690
2	10.04.13	2.57	21.7	84.4	28.1	642
3	30.04.13	2.70	24.6	91.1	27.6	876
4	22.05.13	2.42	17.4	71.9	35.6	1'208
5	21.06.13	1.92	15	77.3	33.3	1'734

Discussion & conclusion:

Chronic lymphocytic leukemia (CLL) is characterized by the clonal proliferation of neoplastic mature lymphocytes in peripheral blood [1]. The real prevalence of leukemia in the dog is not completely known but acute leukemia seem to be more common compared to the chronic form [2]. T-cell phenotype is the most common in canine CLLs [1, 3, 4]. CLL occurs in middle-aged to old animals [3]. It is still not clear if any breed or sex predisposition is present [3]. In the blood smear, the neoplastic population is characterized by small to medium lymphocytes (very similar to normal lymphocytes) with round to oval to slightly indented nuclei, with clumped chromatin and scanty basophilic cytoplasm. Dogs with CLL are often asymptomatic and the diagnosis of leukemia is a casual finding during routine health control of geriatric patient or before surgery. Some dogs however may present lethargy, anorexia and progressive weight loss, polyuria/polydipsia. Sporadic episodes of vomitus were also reported. Clinical examination often reveals splenomegaly and more rarely hepatomegaly. If anemia is present, mucous membranes may be pale. Lymphadenopathy could also be founded. Hematological findings are characterized by leukocytosis and concomitant mild to moderate normochromic normocytic anemia without regeneration [4], although mild regeneration may be present [3, 5]. Neutrophilia and thrombocytopenia were occasionally reported [6, 7]. The absolute number of the lymphocytes may fluctuate in the peripheral blood during CLL but an inexorable progression of the disease is expected to occur. Different leukocyte counts were reported in literature during CLL, ranging from moderate to extreme leukocytosis. A study of 22 cases reported lymphocyte values between 6’000-100’000/µL in CLL [5]; another study on 15 dogs reported ranges from 20’000 to 200’000 lymphocytes/µL [6]. Additionally, lymphocytes between 15’000 and 1’600’000 lymphocytes/µL were reported in a study of 73 dogs with CLL [4]. Immunophenotype of neoplastic cells seems to predict survival in dogs [3]. Dogs with T-CLL seemed to have higher probability of survival than dogs with B-CLL and atypical CLL [3]. Atypical CLL are identified by flow cytometry. Atypical presentation includes cases with CD3-CD8+ immunophenotype, double negative (CD3+CD8-CD4-) or double positive (CD3+CD8+CD4+) T-cell immunophenotype or most rare cases of biphenotype (CD3+CD21+) [3]. Chemotherapy is not always indicated because CLL has an indolent course in most cases [8]. The use of chemotherapy appears to be beneficial in case of unfavorable evolution of CLL. The transformation of CLL in progressive active disease is often characterized by cytopenia, extreme lymphocytosis and concomitant lymphoadenopathy and splenomegaly.

We reported a case of extreme leukocytosis with concomitant normochromic normocytic anemia. Hematological findings and blood smear examination suggested a diagnosis of CLL. The lack of CD34 expression in flow cytometry further supported this diagnosis. A T-cell immunophenotype (T-CLL) was demonstrated by flow cytometry. However, in comparison with classical T-CLL, both CD8 and CD5 were dimly expressed, suggesting a possible transformation towards a more undifferentiated form of this neoplasia. This transformation was also confirmed by the morphology

of some neoplastic cells. These cells showed a larger size and chromatin decondensation compared to the other neoplastic cells and the normal mature lymphocytes. This atypical pattern is likely related to a more aggressive behavior of the neoplasm in comparison with classical T-CLL.

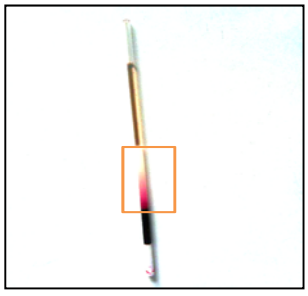
Possible alteration of hematological parameters was suspected in extreme leukocytosis using Sysmex XT-2000iV®. The high number of leukocytes, similar to the erythrocyte count, may have affected the results of the automated analyzer. Manual counts were thus mandatory to prove this possibility and establish the best method to monitor anemia and leukocyte counts.

Sysmex XT 2000 iV® differentiate blood cells by optic and electrical impedance. The impedance method is based on the fact that cells are poor conductors of electricity and if suspended in an electrolyte solution and passed through a small aperture between two electrodes with continuous current, the conductance of the cells is sensed as a change of current, which is proportional to the particle volume. This method allows the discrimination of RBC to WBC. The different volume of these cells can be easily visualized by a tridimensional view of the cells. Despite a similar size on a blood smear, erythrocytes are flat and biconcave while WBC are spherical. The volume of the cells results thus strongly different. In human medicine the volume of RBC and lymphocytes has been estimated and resulted of 89-105 and 194-211 cu μm, respectively [9]. The cut-off values for the cell volume used by Sysmex XT 2000 iV® to discriminate RBC from WBC is not reported.

The optical reading of Sysmex XT 2000 iV® showed a higher number of RBC (RBC-O) compared to the one reported by impedance (RBC-I). Based on the RBC-I, the automated analyzer gave a MCV of 71.1 fL. The RBC-O seemed falsely increased. When the MCV was calculated based on RBC-O (22.9%/4'400'000), a MCV of 52 fL was obtained. A MCV of 52 fL would indicate a microcytic anemia, very unlikely in leukemia, and also not visible on the microscopic evaluation of the blood smear. The optical reading likely overestimated the RBC count which also could have included WBC counts. Indeed, the discrepancy between the automated impedance and the optical count seems to have corresponded to the WBC counts. In addition, these values were in contradiction with the evaluation of blood smear and manual counts. Results obtained by automated impedance were, on the other hand, similar to the manual counts obtained by Bürker chamber. Thus, the results of the automated impedance appeared more reliable than the optical reading.

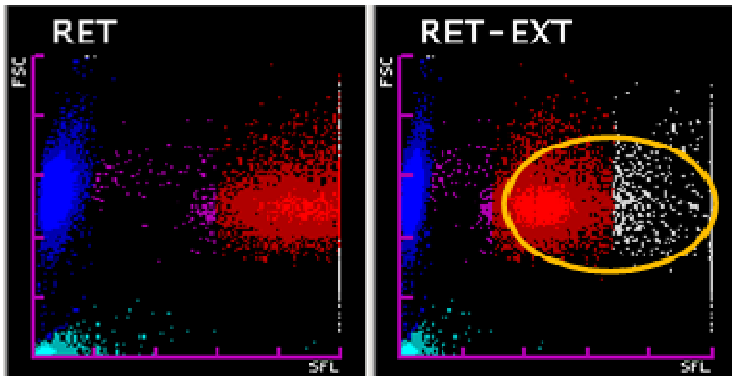
Manual PCV strongly differ from the HCT of the automated analyzer. Since the values of measured MCV appear more accurate than MCV calculated by PCV/RBC manual count, we investigated the source of this discrepancy. Since no signs of microcytosis were present at microscopical evaluation of the blood smear, we considered a possible false underestimation of PCV value due to the incorrect separation of WBC from RBC during centrifugation. This could be due to the very high number of WBC in the blood sample which is confirmed by the presence of a pink hue in the buffy coat (Figure 5, orange square). This hue is often reported as linked to nucleated erythroid cells (metarubricytes) however, metarubricytosis was not identifiable in the present case. It is likely that the extreme leukocytosis impaired correct separation between buffy coat and packed RBC thus causing a false decrease of the PCV.

Figure 5:



For reticulocyte enumeration, Sysmex XT 2000 iV® uses fluorescence and light scattering proprieties. The fluorescent dye polymethine enters through the cell membrane and labels the RNA of reticulocytes, platelets and WBCs and the DNA of nucleated cells. The fluorescence intensity varies among reticulocytes and divides them into three fractions: low, medium and high fluorescence. The sum of the medium and high fluorescence gives the IRF. The IRF increases in dogs with regenerative anemia. In the present case an IRF of 96.9% was identified by the automated analyzer. Interestingly, not all the reticulocyte fractions were increased and a gap was visible between normal RBC and HFR (Figure 6). In blood smear examination, only mild polycromasia and anysocytosis were present. Additionally, the manual reticulocyte count was <1%. An erroneously increased IRF% was thus suspected. It was previously reported that erroneously elevated immature reticulocyte counts (pseudoreticulocytosis) could be detected in leukemic human patients with extreme leukocytosis [10-13]. The same phenomenon was also documented in dogs [14, 15]. The distribution of immature reticulocytes results with an atypical clear gap between the RBC and the reticulocyte regions. This seems to be related to the erroneous interpretation of dead cells, cell fragments containing nucleic acids or altered staining of leukemic cells by Sysmex XT 2000 iV® [14, 15].

Figure 6: Sysmex XT 2000iV® reticulocyte (RET) and extended reticulocyte (RET-EXT) scattergram



In conclusion, Sysmex XT 2000 iV® technology is able to discriminate RBC from WBC in impedance counts in cases of extreme leukocytosis. Manual PCV may provide a false underestimation of PCV value due to incorrect separation of WBC from RBC during centrifugation, giving less reliable values compared to the automated analyzer. Leukocytosis cases with a high reticulocyte count and high IRF have to be carefully considered. Fragments of leukemic cells may be erroneously counted in the reticulocytes regions. RET-EXT scattergrams should be carefully checked and abnormalities confirmed in blood smear examination.

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