Peripheral blood abnormalities and bone marrow infiltration in canine large B-cell lymphoma: is there a link?

V. Martini[1], E. Melzi[1], S. Comazzi[1] and M. E. Gelain[2]

[1]Department of Veterinary Sciences and Public Health, Faculty of Veterinary Medicine, University of Milan, Milan, Italy

[2]Department of Comparative Biomedicine and Food Science, Faculty of Veterinary Medicine, University of Padua, Padua, Italy

Keywords

bone marrow, cytopenia, dog, flow cytometry, lymphoma, staging

Abstract

Official guidelines do not consider bone marrow (BM) assessment mandatory in staging canine lymphoma unless blood cytopenias are present. The aim of this study was to find out if blood abnormalities can predict marrow involvement in canine large B-cell lymphoma. BM infiltration was assessed via flow cytometry. No difference was found between dogs without haematological abnormalities and dogs with at least one. However, the degree of infiltration was significantly higher in dogs with thrombocytopenia, leucocytosis or lymphocytosis and was negatively correlated to platelet count and positively to blood infiltration. Our results suggest that blood abnormalities are not always predictive of marrow involvement, even if thrombocytopenia, leucocytosis or lymphocytosis could suggest a higher infiltration. BM evaluation should therefore be included in routine staging in order not to miss infiltrated samples and to improve classification. However, its clinical relevance and prognostic value are still not defined and further studies are needed.

Correspondence address:

V. Martini, DVM Department of Veterinary Sciences and Public Health Faculty of Veterinary Medicine

University of Milan via Celoria 10

20133 Milan, Italy e-mail:

valeria.martini@unimi.it

Introduction

WHO (World Health Organization) clinical staging system for canine lymphoma is based on the involvement of lymph nodes (stages I– III), spleen and/or liver (stage IV), blood and/or bone marrow (BM) or any other non-lymphoid tissue (stage V).1 In 2010, the Veterinary Cooperative Oncology Group published the official guidelines for response evaluation in dogs with peripheral nodal lymphoma. Under this document, BM evaluation was not required, unless peripheral blood (PB) cytopenias are present suggesting a strong infiltration. In fact, to date there are no standard techniques for quantifying BM involvement, and its prognostic value is still not well defined.2

The reported biological behaviour of lymphoma in different stages is contradictory. On the basis of the results of Flory et al.,3 WHO stage was not associated with prognosis. On the other hand, a recent study has suggested that the contemporary presence of some specific characteristics at the time of diagnosis, among which the absence of BM infiltration, was associated with a 95% probability of >2 years survival; however, the authors did not give a definition of positive and negative BM samples.4 The same research group had previously demonstrated that dogs with stage V lymphoma might benefit from a more aggressive treatment.5 None of these studies, however, investigated the independence of BM infiltration from any other variable with prognostic significance and results should therefore be interpreted with caution. The discrepancy with the results obtained by Flory et al. could be due to the different methods used for BM evaluation (light microscopy versus flow cytometry) as well as to the fact that different lymphoma subtypes had been included in the studies. The use of different methods could also explain the difference among the cutoff used to define a positive sample, ranging from 1 to 20%.3,5

Recent studies have suggested that some morphological and phenotypic features evaluated by flow cytometry could be useful to identify and quantify suspect circulating neoplastic cells in canine large B-cell lymphoma.6,7

The aim of this study was to discover any link between haematological findings and BM infiltration determined by flow cytometry in dogs with large B-cell lymphoma. We hypothesize that BM could be heavily infiltrated even in the absence of PB abnormalities. This would support the advisability to always include BM evaluation in staging procedures.

Materials and methods

Consecutive cases of canine lymphoma were selected from the database of flow cytometric service of Department of Veterinary Sciences and Public Health (Faculty of Veterinary Medicine, University of Milan, Milan, Italy) from March 2006 to Septem- ber 2011. Samples had been sent to the laboratory by private veterinarians for the evaluation of sus- pected lymphoid neoplasm. Inclusion criteria were as follows: (1) a final diagnosis of large B-cell lym- phoma based on microscopic evaluation and flow cytometric immunophenotyping of a fine needle aspirate from a lymph node, and when necessary on histopathology; (2) the availability of a complete blood cell count by automated haematology anal- yser (Sysmex XT-2000iV; Sysmex, Kobe, Japan), at least one good-quality PB smear stained with May-Grunwald-Giemsa, and flow cytometric data of lymph nodes, blood and BM. Lymph node aspi- rates had been collected into RPMI (Sigma Aldrich, St. Louis, MO, USA), blood and BM samples into ethylenediaminetetraacetic acid (EDTA) tubes. All the samples had been sent to the laboratory within 24 h from the collection and immediately processed according to the previously reported protocols.8 Flow cytometry samples had been analysed

using a FACScalibur flow cytometer and CellQuest soft- ware (Becton Dickinson, San Jose, CA, USA). Cells incubated with isotype control antibodies and cells that tested negative for the antigen of interest in samples incubated with anti-canine antibodies were used as negative controls. In addition, the flow cytometer was routinely calibrated using calibration beads (CaliBRITE; Becton Dickinson).

Using flow cytometry, PB and BM infiltration levels were defined as the percentage of CD21+ cells characterized by an elevated forward scatter (FSC-H channel higher than 350), as already described.6 Because a definitive cutoff to classify samples as positive for neoplastic infiltration has never been established, statistical analysis was per- formed using PB and BM infiltration as continuous variables.

PB abnormalities were defined as follows: (1) any haematological value out of internal lab- oratory reference range for the following vari- ables: haemoglobin concentration (<12 g dL−1), platelet count (<100 × 103 µL−1 and/or inade- quate microscopic platelet estimation) or leucocytes (>19.5 × 103 µL−1) based on Sysmex auto- mated analysis, and neutrophils (>11.5 × 103 or <3 × 103 µL−1) or lymphocytes (>4.8 × 103 or <1 × 103 µL−1) based on manual differential count, or (2) the presence of morphologically unclassifiable cells (atypical cells) in the smear, using light microscopy.

Statistics were performed using a stan- dard statistical software (SPSS Statistics 17.0). Mann– Whitney test was performed to analyse the difference in BM infiltration level between dogs with and without anaemia, thrombocytope- nia, leucocytosis, lymphocytosis or atypical cells, and between dogs with and without any abnor- mality at complete blood cell count and/or smear evaluation. The correlation between BM infiltration and haemoglobin concentration, platelet, leuco- cytes, lymphocytes and atypical cells number and PB infiltration was evaluated by means of gener- alized linear models (GLMs), as BM infiltration was found to have a gamma distribution. This test is used to identify correlations between one or more independent variables (either continuous or categorical) and a dependent variable whose distribution is included in the exponential fam- ily (gamma, binomial, multinomial and Poisson, among others).9 For all tests, a P-value <0.05 was considered significant.

Results

From March 2006 to September 2011, 503 canine samples with suspected lymphoid neoplasm were processed in our laboratory and recorded in the database: 412 (81.9%) cases were finally diagnosed as lymphomas, among which 288 (69.9%) had a B- phenotype, while 124 (30.1%) had a T-phenotype. Among B-cell lymphomas, BM flow cytometric data were available only for 84 (29.2%) cases. Twenty- two (26.2%) of these cases were not included in this study because of the lack of data concerning PB, and two (2.4%) because neoplastic cells were small to medium sized. Therefore, 60 dogs fulfilled the inclusion criteria and were included in this study.

Thirty dogs (50%) had at least one of above mentioned PB abnormalities: among these, 17 (56.7%) had a moderate anaemia, 15 (50%) throm-

bocytopenia, 12 (40%) leucocytosis, 11 (36.7%) lymphocytosis, 5 (16.7%) presence of atypical cells, 14 (46.7%) had multiple abnormalities and no one had lymphopenia or granulocytic abnormalities. Atypical cells found were usually medium to large sized, with high nucleus/cytoplasm ratio, round or irregular nucleus with finely reticular chro- matin, occasional prominent nucleoli and slightly basophilic scant to moderate cytoplasm.

Thirty dogs (50%) did not have any haematolog- ical abnormality. Among them, PB infiltration was <5% in 20 (66.7%) cases, between 5 and 10% in 7 (23.3%), between 10 and 20% in 3 (10.0%) and >20% in no one

case. BM infiltration was <5% in 21 (70%) cases, between 5 and 10% in 5 (16.7%), between 10 and 20% in 3 (10%) and >20% in 1 (3.3%) case.

Among the 30 (50%) dogs with at least one PB abnormality, PB infiltration was <5% in 18 (60.0%)cases, between 5 and 10% in 3 (10.0%), between 10 and 20% in 1 (3.3%) and >20% in 8 (26.7%)cases. BM infiltration was <5% in 16 (53.3%) cases, between 5 and 10% in 1 (3.3%), between 10 and 20% in 5 (16.7%) and >20 in 8 (26.7%) cases. Median PB infiltration among all cases was 2.82%, min– max 0.00 – 80.41%. Median BM infiltration among all cases was 3.05%, min– max 0.03 – 74.5%. PB and BM infiltration levels for each case are shown in Fig. 1.

On the basis of Mann– Whitney test, BM infil- tration was significantly higher in dogs with throm- bocytopenia (P = 0.002), leucocytosis (P = 0.032)and lymphocytosis (P = 0.000). On the other hand, there was no significant difference in BM infiltration between dogs with or without anaemia (P = 0.560) and atypical cells (P = 0.360) and between dogs with or without any haematological abnormality (P = 0.237). BM infiltration in groups of dogs based on the presence or absence of PB abnormalities is shown in Table 1.

GLMs showed a significant correlation between BM infiltration and platelet count (P = 0.012) and PB infiltration (P = 0.022): BM infiltration was negatively correlated to platelet count and posi- tively correlated to PB infiltration. No significant correlation was found between BM infiltration and haemoglobin concentration (P = 0.410), leu- cocytes, lymphocytes and atypical cells number (P = 0.223, P = 0.650 and P = 0.185, respectively).

Discussion

Our results highlight that flow cytometric BM infil- tration is negatively correlated to platelet count and positively to blood infiltration, but not to haemoglobin concentration, leucocytes, lympho- cytes and atypical cell number. BM infiltration was different in dogs with or without thrombocytope- nia, leucocytosis and lymphocytosis. Hence, even if a higher BM infiltration should be expected in dogs with one of these three blood abnormalities, its degree cannot be directly predicted by the number of leucocytes or lymphocytes: a heavy BM infiltra- tion can be found both in cases with a slight and a strong lymphocytosis. On the contrary, the lower is the platelet count, the higher is BM infiltration.

On the other hand, no difference in BM infiltration was found between dogs with and without any haematological abnormality.

In human medicine, clinical staging of lym- phoma routinely requires BM evaluation.10 Core biopsy is considered the gold standard technique, but some authors suggested combining it with flow cytometric analysis to improve the sensitivity in neoplastic cells detection.11,12 Under official guide-lines, BM assessment is not required for canine lymphoma unless PB cytopenias are present suggesting a strong infiltration.2 Until now, however, very few data were available regarding the correlation between blood abnormalities and BM involvement. A recent study identified thrombocytopenia and the presence of at least 10% large lymphocytes in blood smears as significant variables to predict BM infiltration: these results, although obtained by morphological evaluation alone, are in according with ours.

No study has been performed correlating blood abnormalities and BM involvement evaluated via flow cytometry, which is a more sensitive and specific test than morphological evaluation alone.14 For this study, we selected large B-cell lymphoma cases, because it is the most common lymphoma subtype in dogs and these neoplastic cells are easily recognizable and quantifiable both in PB and BM by flow cytometry. On the other hand, the staging of small cell lymphoma by flow cytometry could be more challenging, as small

lymphocytes are physiologically present both in PB and BM and are difficult to distinguish from the neoplastic population.14 Moreover, we did not distinguish the different morphological lymphoma subtypes that are grouped together as 'large B-cell lymphoma' as histopathology was available only for a small number of cases. However, studies about the biological behaviour of different morphological

subtypes are still contradictory.15,16

We defined the infiltration level as the percentage of large CD21+ cells in PB and BM samples, because the forward and side scatter of this cells were comparable to those of the neoplastic cells in the lymph node. Unfortunately, to date, no study definitively proving that these are circulating neoplastic cells has been published, but other studies suggested their presence could be due to infiltrating lymphoma.6,7,17,18 These cells could also represent reactive cells, but in our experience, large CD21+ cells account for less than 1% of total nucleated cells in BM from both healthy dogs and dogs with systemic reactive diseases. To our knowledge, large CD21+ cells have not been previously described in BM of dogs without neoplastic conditions, except for a study on BM leucocytes in neonatal dogs in which few large mononuclear CD21+ cells (although less than 1%) were found.19

Among the cases included in this study, no dif- ference in BM infiltration was found between dogs with and without any haematological abnormality. Moreover, even though a clinically useful cutoff has never been defined, it has to be emphasized that around 15% of the dogs without blood abnormalities had a BM infiltration >10% and in one case it was >20%: these cases would be missed if BM was analysed only in dogs with cytopenias, as stated by official guidelines.2 This result suggests that in dogs with a normal complete blood cell count BM status should be assessed, as it is not possible to exclude a strong infiltration only based on PB analysis. BM assessment should therefore be included in routine staging, even if its clinical relevance and prognostic value are not defined, in order not to miss infiltrated samples and to improve classification. However, some abnormalities, such as thrombocytopenia and lymphocytosis, are likely to be suggestive of a higher BM infiltration.

In human large B-cell lymphomas, the low platelet count is correlated to a higher BM involve- ment and is an independent poor prognostic factor.20 Our results and those by Graff et al.13 demonstrate that thrombocytopenia is linked to BM involvement in canine large B-cell lymphoma as well. The low sensitivity (60%) found by Graff et al. highlights that it cannot be ruled out that dogs with a normal platelet count have a strong BM infiltration.

In our study, BM infiltration degree was higher in dogs with leucocytosis or lymphocytosis, even if GLMs did not identify a significant association. As none of the dogs had granulocytic abnormalities, leucocytosis in our cases was likely always due to an increase in lymphoid cells. Moreover, the number of atypical cells was not linked to BM infiltration: this finding could be due to the low number of cases with atypical cells or to the atypical appearance of reactive lymphocytes. Taken together, these results suggest that in the presence of leucocytosis or lymphocytosis a stronger BM infiltration can be expected, while normal leucocyte number and differential count are not sufficient to exclude it.

Anaemia was reported as a negative prognostic factor for dogs with lymphoma21,22; in one of these studies, the authors found no difference in BM cytological involvement between dogs with or without anaemia.21 The absence of association with BM involvement is confirmed by our results and by Graff et al.13 Therefore, anaemia could be due to causes other than BM infiltration and secondary myelophthisis, such as immune-mediated, inflammatory anaemia or other concomitant diseases.

According to GLMs results, a significant correlation was found between PB and BM flow cytometric infiltration. In spite of this, a small number of cases had a strong BM infiltration (>20%) with a slight PB infiltration (<5%) (Fig. 1). Thus, a strong BM infiltration cannot be excluded based on a slight PB infiltration.

Unfortunately, only few cases in this study had a strong BM infiltration (Fig. 1), which could perhaps result in secondary myelophthisis and consequent cytopenias. Therefore, the potential of some blood abnormalities, such as anaemia, to predict BM status could have been underestimated and should be further investigated including a larger number of cases with a strong BM infiltration.

Another pitfall of this study is the lack of clinical information and follow-up data as well as of a common therapeutic approach. This made it impossible to investigate a possible prognostic value of BM infiltration. On the basis of our results, BM should be evaluated in all dogs independently from the presence of peripheral cytopenias in order not to miss infiltrated samples. However, no definitive evidence has been found that BM infiltration level has an influence on prognosis or a protocol- altering decision value. However, quantifying PB and BM infiltration by neoplastic cells at the time of diagnosis and during or after chemotherapy could be a useful tool for monitoring the response to therapy.

In conclusion, this study recommends evaluating BM in all dogs with large B-cell lymphoma in order not to miss infiltrated samples. However, if thrombocytopenia, leucocytosis, lymphocytosis or PB infiltration are present, a heavier BM involvement can be foreseen. It has to be underlined that clinical evidence of prognostic value of BM infiltration is still lacking.

Further clinical studies are needed including more cases and follow-up information to establish the prognostic value of BM involvement and its influence on survival, recurrence and response to therapy. Finally, a similar approach should be attempted in other lymphoma subtypes to better understand the different biological behaviours and to tailor therapy for each lymphoma subtype.

Acknowledgements

The authors wish to thank all the private vets who submitted their clinical samples to their institute for routine examination. This study was supported by Progetti di Ricerca di Interesse Nazionale (PRIN 2008), Ministry of Education, University and Research, Italy.

References

1. Vail DM and Young KM. Canine lymphoma and lymphoid leukemia. In: Withrow and MacEwen's Small Animal Clinical Oncology, 4th edn., SJ Withrow and DM Vail, eds., St. Louis, WB Saunders Co, 2007: 699 – 712.

2. Vail DM, Michels GM, Khanna C, Selting KA, London CA and the Veterinary Cooperative Oncology Group. Response evaluation criteria for peripheral nodal lymphoma in dogs (v1.0) – a Veterinary Cooperative Oncology Group (VCOG) consensus document. Veterinary and Comparative Oncology 2010; 8: 28 – 37.

3. Flory AB, Rassnick KM, Stokol T, Scrivani PV and Erb HN. Stage migration in dogs with lymphoma. Journal of Veterinary Internal Medicine 2007; 21: 1041 – 1047.

4. Marconato L, Stefanello D, Valenti P, Bonfanti U, Comazzi S, Roccabianca P, Caniatti M, Romanelli G, Massari F and Zini E. Predictors of long-term survival in dogs with high-grade multicentric lymphoma. Journal of the American Veterinary Medical Association 2011; 238: 480 – 485.

5. Marconato L, Bonfanti U, Stefanello D, Lorenzo MR, Romanelli G, Comazzi S and Zini E. Cytosine arabinoside in addition to VCAA-based protocols for the treatment of canine lymphoma with bone marrow involvement: does it make the difference? Veterinary and Comparative Oncology 2008; 6: 80 – 89.

6. Williams MJ, Avery AC, Lana SE, Hillers KR, Bachand AM and Avery PR. Canine lymphoproliferative disease characterized by lymphocytosis: immunophenotypic markers of prognosis. Journal of Veterinary Internal Medicine 2008; 22: 596 – 601.

7. Rao S, Lana S, Eickhoff J, Marcus E, Avery PR,Morley PS and Avery AC. Class II major histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. Journal of Veterinary Internal Medicine 2011; 25: 1097 – 1105.

8. Gelain ME, Mazzilli M, Riondato F, Marconato L and Comazzi S. Aberrant phenotypes and quantitative antigen expression in different subtypes of canine lymphoma by flow cytometry. Veterinary Immunology and Immunopathology 2008; 121(3– 4): 179 – 188.

9. Dobson AJ and Barnett A. An Introduction to Generalized Linear Models. 3rd edn., Boca Raton, Chapman and Hall/CRC Press, 2008.

10. Zelenetz AD, Abramson JS, Advani RH, Andreadis CB, Bartlett N, Bellam N, Byrd JC, Czuczman MS, Fayad LE, Glenn MJ, Gockerman JP, Gordon LI, Harris NL, Hoppe RT, Horwitz SM, Kelsey CR, Kim YH, LaCasce AS, Nademanee A, Porcu P, Press O, Pro B, Reddy N, Sokol L, Swinnen LJ, Tsien C, Vose JM, Wierda WG, Yahalom J and Zafar N. Non-Hodgkin's lymphomas. Journal of the National Comprehensive Cancer Network 2011; 9: 484 – 560.

11. Hanson CA, Kurtin PJ, Katzmann JA, Hover JD, Li CY, Hodnefield JM, Meyers CH, Habermann TM and Witzig TE. Immunophenotypic analysis of peripheral blood and bone marrow in the staging of B-cell malignant lymphoma. Blood 1999; 94:3889 – 3896.

12. Talaulikar D, Shadbolt B, Dahlstrom JE and McDonald A. Routine use of ancillary investigations in staging diffuse large B-cell lymphoma improves the International Prognostic Index (IPI). Journal of Hematology & Oncology 2009; 2: 49.

13. Graff E, Spangler E, Smith A, Brauss M and Denhre M. Hematologic abnormalities associated with the presence of lymphoma in the bone marrow of dogs [abstract 12]. Veterinary Clinical Pathology 2011; 40: 603.

14. Comazzi S and Gelain ME. Use of flow cytometric immunophenotyping to refine the cytological

diagnosis of canine lymphoma. The Veterinary Journal 2011; 188: 149 – 155.

15. Kiupiel M, Teske E and Bostock D. Prognostic factors for treated canine malignant lymphoma. Veterinary Pathology 1999; 36: 292 – 300.

16. Ponce F, Magnol JP, Ledieu D, Marchal T, Turinelli V, Chalvet-Monfray K and Fournel-Fleury C. Prognostic significance of morphological subtypes in canine malignant lymphomas during chemotherapy. The Veterinary Journal 2004; 167: 158 – 166.

17. Grindem CB, Page RL, Ammerman BE and Breitschwerdt EB. Immunophenotypic comparison of blood and lymph node from dogs with lymphoma. Veterinary Clinical Pathology 1998; 27: 16 – 20.

18. Joetze AE, Eberle N, Nolte I, Mischke R and Simon D. Flow cytometric evaluation of peripheral blood and bone marrow and fine-needle aspirate samples from multiple sites in dogs with multicentric lymphoma. American Journal of Veterinary Research 2012; 73: 884 – 893.

19. Faldyna M, Sinkora J, Knotigova P, Rehakova Z, Moravkova A and Toman M. Flow cytometric analysis of bone marrow leukocytes in neonatal dogs. Veterinary Immunology and Immunopathology 2003; 95: 165 – 176.

20. Chen LP, Lin SJ and Yu MS. Prognostic value of platelet count in diffuse large B-cell lymphoma. Clinical Lymphoma, Myeloma & Leukemia 2012; 12: 32 – 37.

21. Abbo AH and Lucroy MD. Assessment of anemia as an independent predictor of response to chemotherapy and survival in dogs with lymphoma: 96 cases (1993 – 2006). Journal of the American Veterinary Medical Association 2007; 231: 1836 – 1842.

22. Miller AG, Morkley PS, Rao S, Avery AC, Lana SE and Olver CS. Anemia is associated with decreased survival time in dogs with lymphoma. Journal of Veterinary Internal Medicine 2009; 23: 116 – 122.

Figure 1. Flow cytometric blood (white columns) and bone marrow (BM, black columns) infiltration in 60 dogs with large B-cell lymphoma (percentage of CD21+ cells with a forward scatter higher than 350). A significant correlation was found between blood and BM infiltration level (P = 0.022). Interestingly, however, in some cases, involvement was noticeably higher in BM than in blood.

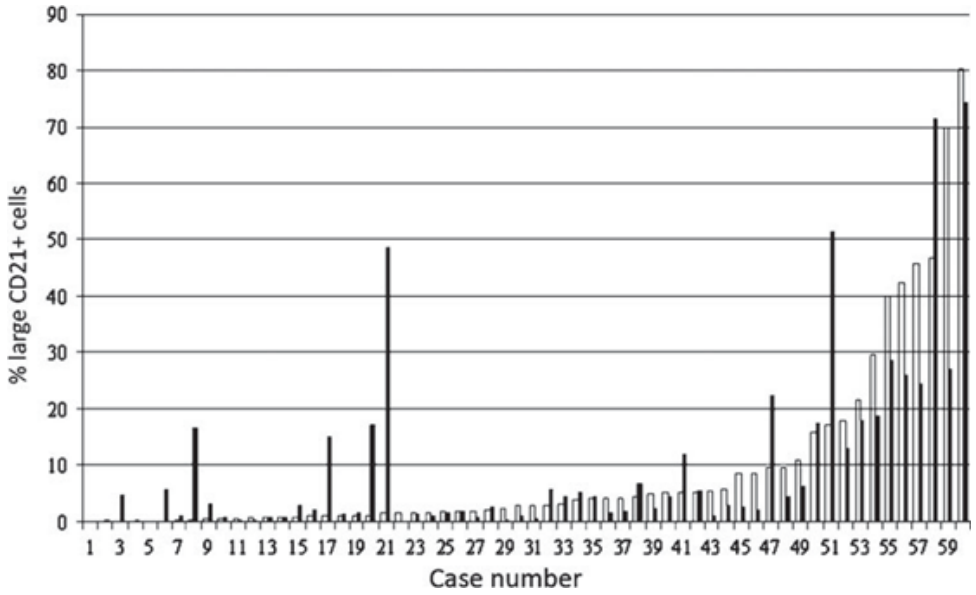

Table 1. Bone marrow (BM) infiltration in different groups of dogs with large B-cell lymphoma, based on the presence or absence of abnormalities at complete blood cell count and/or blood smear evaluation

Peripheral blood abnormality	Number of cases	BM infiltration (%)	
		Median	Min–max
Low haemoglobin concentration	17 (28.3%)	3.03	0.3–74.5
Normal haemoglobin concentration	43 (71.7%)	3.07	0.03–48.62
Low platelet count[ab]	15 (25%)	18	0.35–74.5
Normal platelet count	45 (75%)	2.49	0.03–27
High leucocyte number[a]	12 (20%)	21.55	0.34–74.5
Normal leucocyte number	48 (80%)	2.58	0.03–51.42
High lymphocyte number[a]	11 (18.3%)	24.43	1.2–74.5
Normal lymphocyte number	49 (81.7%)	2.2	0.03–51.42
Presence of atypical cells	5 (8.3%)	1.35	0.3–74.5
Absence of atypical cells	55 (91.7%)	3.25	0.03–71.26
Any abnormality	30 (50%)	3.97	0.13–74.5
No abnormalities	30 (50%)	2.58	0.03–22.4

[a]Significant difference in BM infiltration based on Mann–Whitney test ($P \leq 0.05$).
[b]Significant correlation with BM infiltration based on generalized linear models ($P \leq 0.05$).