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PROGNOSTIC FACTORS IN CANINE ACUTE LEUKAEMIAS: A RETROSPECTIVE STUDY

Running headline: prognosis in canine acute leukaemias

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Abstract

Canine acute leukaemias (ALs) have a poor prognosis, with reported survival times (ST) of only a few weeks or months. Also, clinical studies assessing prognostic factors are lacking. The present study aims to retrospectively assess variables that predict ST in dogs with AL, and to identify correlations between outcome and therapeutic protocols. Diagnosis and sub-classification into AL subtypes was made based on haematological findings, morphological assessment and flow cytometric immunophenotyping. Clinical-pathological features of AL subtypes at presentation concurred with those described in the literature. A normal neutrophil count at presentation significantly prolonged ST ($p=0.027$). Additionally, there was a trend for anaemic dogs to have shorter survival compared to those without anaemia and the incorporation of cytosine in the chemotherapy protocol produced a moderate but not significant increase in median ST for dogs with AL. Further prospective studies with standardized treatments are needed to confirm and improve our results.

Introduction

Acute leukaemias (ALs) are not uncommon in dogs. Historically, the diagnosis of different AL subtypes relied only on the morphological and cytochemical analysis. However, the spread of more sophisticated techniques such as flow cytometry has improved the diagnostic workup, improving the classification of immature cells. [1,2]

However, despite advances in classification schemes and diagnostic techniques, no therapeutic improvement has been obtained for canine ALs, and prognosis is still poor, with reported survival times of only a few weeks or months[3]. Effective chemotherapeutic protocols have not been developed in veterinary medicine, and regardless of the administered regime, the disease progresses rapidly. Because of these discouraging clinical features, canine ALs are not the object of large studies assessing prognostic factors, and novel therapeutic protocols are not attempted.

In human medicine, prognostic factors and treatments vary among different AL subtypes. In particular, prognosis for human ALs is mostly predicted by cytogenetic and molecular genetic abnormalities, which stratify patients into different risk groups for each subtype. [4,5] Furthermore, age, high WBC count at presentation, anaemia and phenotype were reported to influence prognosis in specific AL subtypes. [6-10]

The present work had two aims: first to evaluate retrospectively in dogs with AL whether the biological and haematological variables at presentation could predict survival, and second to relate multiple therapeutic protocols to the prognosis.

Materials and methods

Between Jan 2009 and March 2014, the database of the Flow Cytometric Service of the Department of Veterinary Sciences and Public Health (University of Milan, Milan, Italy) was interrogated and all consecutive canine cases with suspected AL were selected. Inclusion criteria were: 1) a final diagnosis of AL, based on the clinical suspicion, smear evaluation and flow cytometric data; and 2) availability of flow cytometric data for re-evaluation, comprising antibody panel shown in table 1. Exclusion criteria were: 1) severe lymphadenomegaly with lymph node cytology having features compatible with lymphoma; 2) lack of data concerning lymph node size at admission. Mild lymphadenomegaly was not considered an exclusion criterion, except for cases showing cytological features suggestive of specific lymphoma subtypes. Flow cytometry (FC) was performed on peripheral blood as previously described. [11] When available, immunophenotype was also obtained from bone marrow samples. All the samples were collected into EDTA tubes and shipped to the Laboratory within 24 hours from collection.

Cases were classified as follows: acute B-cell lymphoid leukaemia (B-ALL) when cells were CD21 and/or CD79a positive and negatively stained for all T-cells and myeloid markers; acute T-cell lymphoid leukaemia (T-ALL) when cells were CD3 and/or CD5, CD4, CD8 positive and negatively stained for all B-cells and myeloid markers; acute myeloid leukaemia (AML) when stained positive for MPO and/or CD11b, CD4, CD14 and negative for all lymphoid markers; acute undifferentiated leukaemia (AUL) when stained negative for all lymphoid and myeloid markers. Positive staining for CD34 was considered suggestive but not conclusive for AL. AMLs were further sub-classified into the 7 French American British (FAB) subgroups [12,13] based on combined morphological assessment and immunophenotype by FC.

Caseload clinical data was obtained from the clinical records and by phone calls to referring veterinarians. Background information collected for each dog included: signalment,

treatment (if any), response to treatment (clinical and haematological), date and cause of death. Haematological abnormalities were defined as values exceeding the laboratory reference interval (RI). Haematological improvement was defined as a trend of any abnormal value to return to RI, whereas haematological worsening was defined as abnormal values further distancing from RI or appearance of new abnormalities. When available, multiple control CBCs were evaluated to assess the trend of haematological values' changes.

Statistical analysis was performed via SPSS 17.0 for Windows. Significance was set at $p \leq 0.05$ for all tests.

A multinomial logistic regression was performed to assess any possible association between AL subgroups (B-ALL, T-ALL, AML and AUL) and the following variables: breed (pure or mixed), sex (male or female), age (< or >10 years), anaemia (present or not), thrombocytopenia (present or not), leukocyte count (within reference interval, leukopenia or leukocytosis), neutrophil count (within reference interval, neutrophilia, neutropenia), lymphocyte count (within reference interval, lymphocytosis, lymphopenia), atypical cells (present or not).

These variables were investigated via Kaplan-Meier curves and Log-Rank test to verify their influence on survival time (ST). ST was defined as the time between diagnosis and death for AL. Cases were censored for survival analysis if still alive at the data analysis closure or if lost to follow-up.

Results

Case description

Seventy-one dogs with AL matched the inclusion criteria. Among them, 20 (28.2%) were classified as B-ALLs, 9 (12.7%) as T-ALLs, 25 (35.2%) as AMLs and 17 (23.9%) as

AULs. AML cases were further classified as myeloblastic without differentiation (AML-M1) in 11 (44%) dogs, as myeloblastic with neutrophilic differentiation (AML-M2) in 1 (4%) dog, as myelomonocytic (AML-M4) in 7 (28%) dogs, as monocytic (AML-M5) in 2 (8%) dogs, as acute erythroid leukaemia (AML-M6a) in 1 (4%) dog, and as megakaryoblastic leukaemias (AML-M7) in 3 (12%) dogs. In 5 cases, comprising 1 B-ALL, 2 T-ALL and 2 AML, the final diagnosis was only obtained by bone marrow analysis due to the absence of circulating neoplastic cells.

Breed was reported for 64 dogs: among them, 50 (78.1%) were pure-breed and 14 (21.9%) were mixed-breed. The most represented breeds were Golden retriever (n=10), German shepherd (n=8), Labrador retriever (n=6) and Doberman (n=4); another 18 breeds were represented by 1 to 3 cases each. Prevalence of mixed- or pure-breed did not vary among the 4 AL subgroups (p=0.192).

Sex was reported for 64 dogs: 35 (54.7%) were females and 29 (45.3%) were males. Prevalence of female or male sex did not vary among the 4 AL subgroups (p=0.477).

Age at diagnosis was reported for 63 dogs. Overall mean age was 7.5±3.5 years (median 8 years, range, 7 months–16 years). In particular, 41 (65.1%) dogs were <10 year-old and 22 (34.9%) were >10 year-old. Graphic representation of age distribution showed a bimodal distribution, with a lower peak at 3 years, and a higher peak at 10 years (fig 1). Prevalence of dogs < or >10 year-old did not vary among the 4 AL subgroups (p=0.085).

CBC at diagnosis was available for 64 dogs. 61 (95.3%) had thrombocytopenia, 58 (90.6%) had anaemia. Mean leukocyte count was 98.73±110.72 10^3/µl (median 60 10^3/µl, range 1.77-571.48 10^3/µl): 45 (70.3%) dogs had leukocytosis and 9 (14.1%) had leukopenia, 50 (78.2%) had neutropenia and 2 (3.1%) had neutrophilia, 46 (71.9%) had lymphopenia and 2 (3.1%) lymphocytosis, 59 (92.2%) had circulating neoplastic cells. Leukocytosis was

always due to the presence of atypical cells. Prevalence of CBC abnormalities did not vary among the 4 AL subgroups, except for WBC count abnormalities, that were significantly different among the 4 AL subgroups (p=0.025). In particular, among B-ALLs, 2 out of 17 (11.8%) dogs had WBC count within reference interval, 2 (11.8%) had leukopenia and 13 (76.5%) had leukocytosis; among T-ALLs, 2 out of 7 (28.6%) had leukopenia and 5 (71.4%) had leukocytosis; among AMLs, 8 out of 25 (32%) had WBC count within reference interval, 4 (16%) had leukopenia, and 13 (52%) had leukocytosis; finally, among AULs, one dog out of 15 (6.7%) had leukopenia and 14 (93.3%) had leukocytosis.

Outcome

Follow-up data were obtained for 38 (53.5%) dogs, including 9 (23.7%) B-ALLs, 6 (15.8%) T-ALLs, 12 (31.6%) AMLs and 11 (28.9%) AULs. In particular, 8 (21.1%) dogs were euthanized immediately after diagnosis; these dogs were excluded from the median ST calculation. Two (5.3%) dogs did not receive any treatment and died after 6 and 7 days from diagnosis, respectively. Ten (26.3%) dogs were treated with corticosteroids. Eighteen (47.4%) dogs were treated with various chemotherapy protocols, with or without the inclusion of corticosteroids. These included single-agent chemotherapy (chlorambucil, L-asparaginase or vincristine) or single-agent tyrosine-kinase inhibitor (TKI) (masitinib) (44.4%), a CHOP-based chemotherapy regimen (33.3%), and different chemotherapy protocols including cytosine arabinoside (22.2%).

Thirteen dogs had their CBC checked after starting treatment. Recheck time varied among cases, depending on referring veterinarians preferences; however, in all cases the first control CBC was performed within 1 week from diagnosis. In 6 (46.2%) cases, haematological values were similar to those obtained at diagnosis: among them, 3 had been

154 treated with corticosteroids alone, and 3 with a combination of corticosteroids and
155 chemotherapy. In 5 (38.5%) dogs, haematological parameters improved: among them, 1 dog
156 was treated with corticosteroids alone, subsequently relapsed when corticosteroids dosage
157 was reduced, and died after 73 days, and 4 dogs received chemotherapy. Finally, in 2 (15.4%)
158 cases haematological values worsened after chemotherapy treatment.

159 Median ST for the 30 treated and untreated cases which were not immediately
160 euthanized was 9 days (range, 1-120 days). At data analysis closure, only one dog was still
161 alive, after 90 days: although morphological evaluation of neoplastic cells suggested a
162 lymphoid lineage, their lineage could not be confirmed by flow cytometry, leading to a final
163 diagnosis of AUL; CBC at diagnosis showed leukocytosis, anaemia and thrombocytopenia;
164 the dog was treated with corticosteroids and CHOP-based chemotherapy and haematological
165 parameters normalized within a few days.

166 Median ST (treated and untreated) was 8 days (range, 5-46 days) for B-ALLs, 10 days
167 for T-ALLs and AMLs (range, 4-120 days and 3-73 days, respectively) and 7 days (range, 1-
168 90 days) for AULs.

169 When considering signalment, median ST (treated and untreated) was 8 days (range,
170 3-120 days) for pure-breed dogs (B-ALL n=3, T-ALL n=5, AML n=8, AUL n=6) and 15 days
171 (range, 1-46 days) for mixed-breed dogs (B-ALL n=2, AML n=3, AUL n=2), 10 days (range,
172 4-120 days) for females (B-ALL n=3, T-ALL n=4, AML n=5, AUL n=4) and 7 days (range,
173 1-40 days) for males (B-ALL n=1, T-ALL n=1, AML n=6, AUL n=4), 7 days (range, 1-120
174 days) for dogs <10 year-old (B-ALL n=5, T-ALL n=4, AML n=7, AUL n=4) and 10 days
175 (range, 7-90 days) for dogs >10 year-old (T-ALL n=1, AML n=4, AUL n=3).

176 When considering haematology results, median ST (treated and untreated) was 10
177 days for dogs with normal WBC count (B-ALL n=1, AML n=2) and for dogs with leukopenia

(B-ALL n=1, T-ALL n=2, AML n=2) (range, 8-73 days and 4-46 days, respectively) and 7 days (range, 1-120 days) for dogs with leukocytosis (B-ALL n=3, T-ALL n=3, AML n=7, AUL n=8), 60 days (range, 3-120 days) for dogs with neutrophil count within RI (T-ALL n=2, AML n=2, AUL n=1), 7 days (range, 1-90 days) for dogs with neutropenia (B-ALL n=4, T-ALL n=3, AML n=9, AUL n=6) and 1 and 5 days respectively for the two dogs with neutrophilia (AUL and B-ALL, respectively), 6 days (range, 1-73 days) for dogs with lymphocyte count within RI (B-ALL n=1, T-ALL n=1, AML n=2), 7 days (range, 1-120 days) for dogs with lymphopenia (B-ALL n=3, T-ALL n=4, AML n=9, AUL n=8) and 5 days for the only dog with lymphocytosis (B-ALL), 7 days (range, 1-120 days) for dogs with atypical cells in the blood smear (B-ALL n=5, T-ALL n=5, AML n=9, AUL n=8) and 7 and 28 days respectively for the two dogs without atypical cells in the blood smear (AML n=2) 60 days (range, 3-120 days) for dogs without anaemia (T-ALL n=1, AML n=1, AUL n=1) and 9 days (range, 1-90 days) for anaemic dogs (B-ALL n=5, T-ALL n=4, AML n=10, AUL n=7). The only dog (B-ALL) with normal platelet count died after 8 days, whereas median survival time for thrombocytopenic dogs (B-ALL n=4, T-ALL n=5, AML n=11, AUL n=8) was 9 days (range, 1-120 days).

When considering treatment, median ST was 10 days (range, 7-73 days) for dogs treated with corticosteroids (B-ALL n=2, T-ALL n=1, AML n=4, AUL n=3), and 9 days (range, 1-90 days) for dogs treated with chemotherapy (B-ALL n=3, T-ALL n=3, AML n=7, AUL n=5). In particular, median ST was 5 days (range, 1-60 days) for dogs treated with single-agent chemotherapy or single agent TKI (B-ALL n=1, T-ALL n=1, AML n=3, AUL n=4), 11 days (range, 5-90 days) for dogs receiving a CHOP-based chemotherapy protocol (B-ALL n=1, T-ALL n=1, AML n=2, AUL n=1), and 40 days (range, 9-120) for dogs receiving any chemotherapy protocol including cytosine arabinoside (B-ALL n=1, T-ALL n=1, AML n=2).

When considering recheck CBCs, median ST (treated and untreated) was 22 days (range, 9-46 days) for dogs with stable haematological values (B-ALL n=1, AML n=3, AUL n=1) and 36 days (range, 5-90 days) for dogs experiencing a haematological improvement (B-ALL n=1, T-ALL n=1, AML n=2, AUL n=1). The two dogs characterized by worsening of haematological values died after 39 and 60 days, respectively (AML and AUL, respectively).

None of the investigated variables significantly influenced ST, with the exception of neutrophil count, as dogs with neutrophil count within RI survived significantly longer than dogs with neutropenia and neutrophilia (p=0.027).

Discussion

Canine AL is an aggressive type of cancer that progresses rapidly despite treatment. Also, the treatment of canine AL remains largely unsatisfactory despite a general improvement in chemotherapy and supportive care. [3] The present study describes the clinical-pathological features of canine ALs at diagnosis and further investigates several factors for prediction of ST.

Based on our results, signalment and haematological values on presentation did not differ among B-ALLs, T-ALLs, AMLs and AULs, with the exception of WBC count: indeed, although leukocytosis was the most common finding for all AL subtypes, dogs with AML tended to have a normal WBC count more frequently than all the other subgroups.

According to the literature, only two studies reported the clinical and clinical-pathological features of confirmed canine leukaemias but no data on the clinical follow-up were reported. [1,2]

The study by Adam and colleagues [1] included ALLs, AMLs and chronic lymphocytic leukaemias (CLLs). The proportion of AML and ALL cases was similar to our results, whereas AULs were not considered. A possible explanation might be related to a wider antibody panel used in this study: the authors included antibodies reacting against cytoplasmic CD3 (able to identify T-ALLs staining negative for all surface markers), and against four different isoforms of CD11 (whereas we only tested CD11b). Similarly, Tasca and colleagues [2] did not report AULs. However, in this study the diagnosis of AML was only based on the cellular positive staining for CD34 and CD45, and negative staining for CD3 and CD79a. Since the myeloid lineage was not definitively proven, a possible misclassification of some AUL as AML might have occurred.

Also, in this latter study, CD34 was used to diagnose AL and rule out CLL and leukemic lymphomas, whereas in the present study and in the one by Adam and colleagues [1] the final diagnosis was obtained combining clinical data, morphological evaluation and immunophenotype. CD34 expression was considered suggestive but not conclusive for AL. CD34 is exclusively expressed by early precursors, thereby being regarded as a marker of AL [14,15] and associated to a short survival in dogs with neoplastic lymphocytosis. [16] However, CD34 expression has been described in a subset of canine lymphoma, [17] and CD34negative ALs have been also reported. [18-22] Therefore, the expression of CD34 by itself should not be used to confirm or exclude a diagnosis of AL.

Despite the different inclusion and diagnostic criteria, epidemiological data obtained in the present study overlap those reported in literature. [1,2] Indeed, in all three studies, many different breeds were represented, with a prevalence of large and giant breeds, such as German Shepherds and Retrievers. In particular, one of the already published studies [1] found a significant over-representation of Golden Retrievers in the ALL group compared to control

population. Age at diagnosis was similar among the three groups, and no significant difference in sex among AL subtype could be identified by any study.

The frequency of ALLs and AMLs was about equal in all three studies, with B-ALLs more common than T-ALLs, whereas the frequency of specific AML subtypes widely varied among the three studies, most likely because of the different methods used for the sub-classification. Frequency of anaemia and thrombocytopenia did not differ among AL subtypes in any study. In contrast, a subtle difference in WBC count among AL subtypes was found in the present study, but was not statistically significant. This discrepancy might be related to the inclusion in the present study of aleukaemic leukaemias, in which the diagnosis was made based on a bone marrow sample.

To our knowledge, this is the first study investigating possible prognostic factors for canine AL; however, only neutrophil count differed significantly. In addition, there was a trend for anaemic dogs to have a shorter ST than dogs without anaemia (median ST, 9 versus 60 days), suggesting a possible prognostic role for anaemia. One hypothesis is that the reduced number of dogs with follow-up data and the huge variety of treatment protocols adopted have strongly influenced the survival analysis. Furthermore, the paucity of significant results may be associated to the overall short ST in our study. At the same time, these factors may have lead to an over-estimation of the prognostic value of the neutrophil count, as only few dogs presented with neutrophil count within RI or neutrophilia.

. When leukaemia is diagnosed, peripheral cytopenias are mostly caused by myelophthisis and new blood cells are not produced in sufficient number to replenish those destroyed because of aging. Therefore, a neutrophil count within RI, which is associated with a better prognosis based on our results, may document an early diagnosis. Conversely, erythrocytes have a longer lifespan compared to leukocytes and platelets, and anaemia can

occur later in such cases. Thus, the shorter survival of anaemic dogs could be due to a delay in the diagnosis from the onset of neoplasia, more than to a higher aggressiveness of the tumour itself.

In addition, when considering treatment, although not significant, the incorporation of cytosine arabinoside tended to prolong survival compared to the other regimens described here. Cytosine arabinoside has substantial antileukaemic activity and is the mainstay in primary treatment regimens for human ALs, mainly for the non-lymphoblastic leukemias. According to the literature, the use of cytosine in combination with an anthracycline for the treatment of human ALs leads to long-term overall survival. [23,24]

Experience in the treatment of canine AL is limited because of the low incidence, the aggressiveness of the disease, and the typical poor clinical condition of affected dogs at presentation. One study has been published by our research group, supporting the role of cytosine administered as a continuous intravenous infusion in addition to standard CHOP-based chemotherapy in dogs with leukaemic lymphoma. [25] Three out of the 4 dogs treated with cytosine in the current study were among those that survived the longest (data not shown). These preliminary results warrant further confirmation in future randomized studies to define the efficacy and cost-effectiveness of cytosine incorporated in standard protocols.

Only a few cases in the present study achieved clinical and/or haematological remission. This is in agreement with what is reported in the veterinary literature. [3] On the contrary, complete remission is achieved in up to 80% cases in human medicine, depending on AL subtype, patient age at diagnosis and other prognostic factors. [5] This difference could be due to a more aggressive behaviour of canine ALs compared to human ALs, or to a delay in the diagnosis. Further studies are needed to assess if is there any dissimilarity in

296 cytogenetic and molecular genetic abnormalities underlying neoplasia between canine and
297 human ALs, which could further explain the different response to first treatment.

298 The retrospective nature of this case series is a limitation of the present study: the
299 treatment protocol was not randomised, since therapy options were mainly related to the
300 discretion of the owners and the attending veterinarians. The dogs' clinical status and
301 expected prognosis may have also influenced the selection of a specific treatment, as it is
302 possible that dogs with worse clinical conditions were less likely to receive treatment.
303 Additionally, the paucity of statistical significance could be attributed to the huge variety of
304 treatment regimens adopted, the inclusion of all types of ALs, and the lack of molecular
305 analysis investigating FLT3, RAS and C-KIT mutations. These mutations have a prognostic
306 role in human ALs and have been previously reported in canine ALs, [26,27] but the prognostic
307 role in this species has never been investigated. At the same time, the significant survival
308 improvement related to the neutrophil count may have been influenced by these limitations
309 and should be confirmed in further studies.

310 In conclusion, neutrophil count and anaemia are the only variable apparently
311 associated with prognosis in canine ALs and the incorporation of cytosine seemed promising
312 for dogs with AL. Further prospective studies with standardized therapies are needed, to
313 confirm and complete our results.

314

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References

1. Adam F, Villiers E, Watson S, Coyne K and Blackwood L. Clinical pathological and epidemiological assessment of morphologically and immunologically confirmed canine leukaemia. *Veterinary and Comparative Oncology* 2009; **7(3)**: 181-195.

2. Tasca S, Carli E, Caldin M, Menegazzo L, Furlanello T and Solano Gallego S. Hematologic abnormalities and flow cytometric immunophenotyping results in dogs with hematopoietic neoplasia: 210 cases (2002-2006). *Veterinary Clinical Pathology* 2009; **38(1)**: 2-12.

3. Vail DM, Pinkerton Me and Young KM. Canine lymphoma and lymphoid leukemias. In: *Withrow and MacEwen's Small Animal Clinical Oncology*. 5th edn., St Louis, Saunders Elseviers, 2012: 608-638.

4. Cornell RF and Palmer J. Adult acute leukaemia. *Disease-a-month : DM* 2012; **58(4)**: 219-238.

5. Pui CH, Carroll WL, Meshinchi S and Arceci RJ. Biology, risk stratification, and therapy of paediatric acute leukaemias: an update. *Journal of Clinical Oncology* 2011; **29(5)**: 551-565.

6. Hoelzer D, Thiel E, Löffler H Büchner T, Ganser A, Heil G et al. Prognostic factors in a multicenter study for treatment of acute lymphoblastic leukaemia in adults. *Blood* 1988; **71(1)**: 123-131.

7. Smith M, Arthur D, Camitta B, Carroll AJ, Crist W, Gaynon P et al. Uniform approach to risk classification and treatment assignment for children with acute lymphoblastic leukemia. *Journal of Clinical Oncology* 1996: **14(1)**: 18-24.

8. Rowe JM, Buck G, Burnett AK, Chopra R, Wiernik PH, Richards SM et al. Induction therapy for adults with acute lymphoblastic leukaemia: results of more than 1500 patients from an international ALL trial: MRC UKALL XII/ECOG. *Blood* 2005; **106(12)**: 3760-3767.

9. Appelbaum FR, Gundacker H, Head DR, Slowak ML, Willman CL, Godwin JE et al. Age and acute myeloid leukaemia. *Blood* 2006; **107**: 3481-3485.

10. Teuffel O, Stanulla M, Cario G, Ludwig WD, Rottgers S, Schafer BW et al. Anaemia and survival in childhood acute lymphoblastic leukaemia. *Haematologica* 2008; **93(11)**: 1652-1657.

11. 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.

12. Jain MC, Blue JT, Grindem CB, Harvey JW, Kociba GJ, Krehbiel JD et al. Proposed criteria for classification of acute myeloid leukemia in dogs and cats. *Veterinary Clinical Pathology* 1991; **20(3)**: 63-82.

13. McManus PM. Classification of myeloid neoplasms: a comparative review. *Veterinary Clinical Pathology* 2005; **34(3)**: 189-212.

14. Vernau W and Moore PF. An immunophenotypic study of canine leukaemias and preliminary assessment of clonality by polymerase chain reaction. *Veterinary Immunology and Immunopathology* 1999; **69(2-4)**: 145-164.

15. Workman HC and Vernau W. Chronic lymphocytic leukaemia in dogs and cats: the veterinary perspective. *The Veterinary Clinics of North America. Small Animal Practice* 2003; **33(6)**: 1379-1399.

16. 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(3)**: 596-601.

17. Rao S, Lana S, Eickhoff J, Marcus E, Avery PR, Morley PS et al. Class II major Histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. *Journal of Veterinary Internal Medicine* 2011; **25(5)**: 1097-1105.

18. Ameri M, Wilkerson MJ, Stockham SL, Almes KM, Patton KM and Jackson T. Acute megakaryoblastic leukaemia in a German Shepherd dog. *Veterinary Clinical Pathology* 2010; **39(1)**: 39-45.

19. Comazzi S, Gelain ME, Bonfanti U and Roccabianca P. Acute megakaryoblastic leukaemia in dogs: a report of three cases and review of the literature. *Journal of the American Animal Hospital Association* 2010; **46(5)**: 327:335.

20. Tomiyasu H, Fujino Y, Takahashi M, Ohno K and Tsujimoto H. Spontaneous acute erythroblastic leukaemia (AML-M6Er) in a dog. *The Journal of Small Animal Practice* 2011; **52(8)**: 445-447.

21. Valentini F, Tasca S, Gavazza A and Lubas G. Use of CD9 and CD61 for the characterization of AML-M7 by flow cytometry in a dog. *Veterinary and Comparative Oncology* 2011; **10(4)**: 312-318.

22. Mylonakis ME, Kritsepi-Konstantinou M, Vernau W, Valli VE, Pardali D and Koutinas AF. Presumptive pure erythroid leukaemia in a dog. *Journal of Veterinary Diagnostic Investigation* 2012; **24(5)**: 1004-1007.

23. Kern W and Estey EH. High-dose cytosine arabinoside in the treatment of acute myeloid leukaemia: Review of three randomized trials. *Cancer* 2006; **107(1)**: 116-24.

24. Bishop JF, Matthews JP, Young GA, Szer J, Gillett A, Joshua D et al. A randomized study of high-dose cytarabine in induction in acute myeloid leukaemia. *Blood* 1996; **87(5)**: 1710–1717.

25. Marconato L, Bonfanti U, Stefanello D, Lorenzo MR, Romanelli G, Comazzi S et al. 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(2)**: 80-89.

26. Usher SG, Radford AD, Viliers EJ and Blackwood L. RAS, FLT3 and C-KIT mutations in immunophenotyped canine leukemias. *Experimental hematology* 2009; **37(1)**: 65-77.

27. Suter SE, Small GW, Seiser EL, Thomas R, Breen M and Richards KL. FLT3 mutations in canine acute lymphocytic leukemia. *BMC Cancer* 2011; **11**: 38.

404 Table 1: antibodies used for the flow cytometric immunophenotyping of neoplastic cells in 71
405 dogs with acute leukaemia

Target molecule	**Antibody clone**	**Source**	**Specificity**
CD45	YKIX716.13	Serotec, Oxford, UK	All leukocytes
CD3	CA17.2A12	Serotec	T-cells
CD5	YKIX322.3	Serotec	T-cells
CD4	YKIX302.9	Serotec	T-helper cells and neutrophils
CD8	YCATE55.9	Serotec	T-cytotoxic cells
CD21	CA2.1D6	Serotec	Mature B-cells
CD79a	HM57	Serotec	B-cells
CD11b	M1/70	eBioscience, San Diego, CA, USA	Myeloid cells
CD14	TUK4	Serotec	Monocytes
MPO	2C7	Serotec	Myeloid cells
CD34	1H6	BD Pharmingen	Precursors

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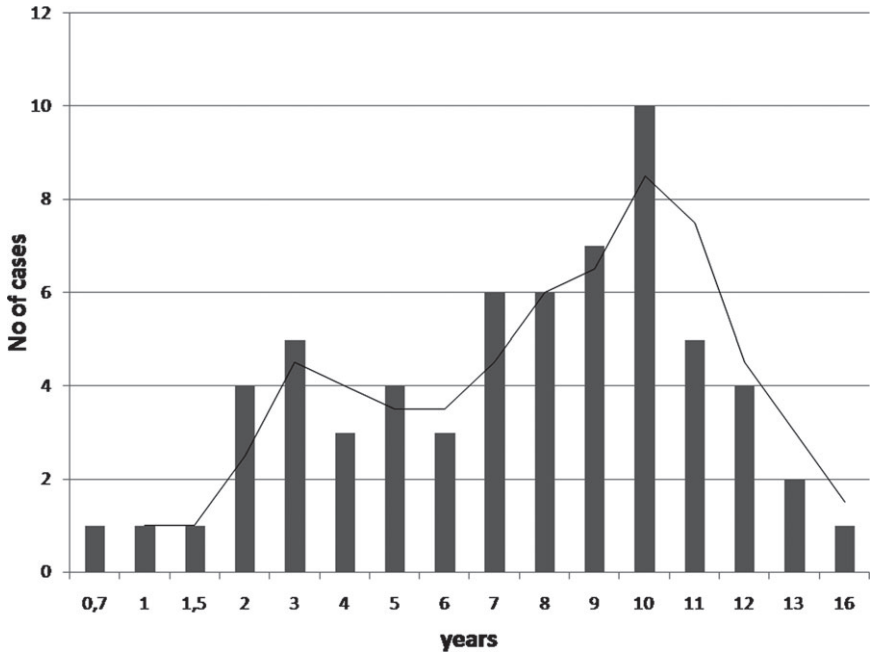
408 Figure 1: age distribution of 63 dogs diagnosed with acute leukaemia.



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