Prognostic factors in canine acute leukaemias: a retrospective study

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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. This 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 with 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.

Keywords
acute leukaemia, anaemia, cytosine, flow cytometry, neutrophil count, prognosis

Introduction
Acute leukaemias (ALs) are not uncommon in dogs. Historically, the diagnosis of different AL subtypes relied only on the morphological and cytochemical analyses. However, the spread of more sophisticated techniques such as flow cytometry (FC) has improved the diagnostic workup and also 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 (STs) 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

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

Materials and methods
Between January 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...
Table 1. Antibodies used for the flow cytometric immunophenotyping of neoplastic cells in 71 dogs with AL

<table>
<thead>
<tr>
<th>Target molecule</th>
<th>Antibody clone</th>
<th>Source</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>YKIX716.13</td>
<td>Serotec (Oxford, UK)</td>
<td>All leukocytes</td>
</tr>
<tr>
<td>CD3</td>
<td>CA17.2A12</td>
<td>Serotec</td>
<td>T cells</td>
</tr>
<tr>
<td>CD5</td>
<td>YKIX322.3</td>
<td>Serotec</td>
<td>T cells</td>
</tr>
<tr>
<td>CD4</td>
<td>YKIX302.9</td>
<td>Serotec</td>
<td>T-helper cells and neutrophils</td>
</tr>
<tr>
<td>CD8</td>
<td>YCATE55.9</td>
<td>Serotec</td>
<td>T-cytotoxic cells</td>
</tr>
<tr>
<td>CD21</td>
<td>CA2.1D6</td>
<td>Serotec</td>
<td>Mature B cells</td>
</tr>
<tr>
<td>CD79a</td>
<td>HMS7</td>
<td>Serotec</td>
<td>B cells</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>eBioscience (San Diego, CA, USA)</td>
<td>Myeloid cells</td>
</tr>
<tr>
<td>CD14</td>
<td>TUK4</td>
<td>Serotec</td>
<td>Monocytes</td>
</tr>
<tr>
<td>MPO</td>
<td>2C7</td>
<td>Serotec</td>
<td>Myeloid cells</td>
</tr>
<tr>
<td>CD34</td>
<td>1H6</td>
<td>BD Pharmingen (San Diego, CA, USA)</td>
<td>Precursors</td>
</tr>
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interrogated, and all consecutive canine cases with suspected AL were selected. Inclusion criteria were as follows: (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 as follows: (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. FC was performed in peripheral blood as previously described. When available, immunophenotype was also obtained from bone marrow samples. All the samples were collected into ethylenediaminetetraacetic acid (EDTA) tubes and shipped to the laboratory within 24 h 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 myeloperoxidase (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 subclassified into the seven French American British (FAB) subgroups based on combined morphological assessment and immunophenotype using FC.

Caseload clinical data were obtained from the clinical records and by phone calls to the 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), leucocyte count (within RI, leucopenia or leucocytosis), neutrophil count (within RI, neutrophilia or neutropenia), lymphocyte count (within RI, lymphocytosis or lymphopenia) and atypical cells (present or not).

These variables were investigated using Kaplan–Meier curves and log-rank test to verify their influence on 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 five cases, comprising B-ALL in one, T-ALL in two and AML in two, the final diagnosis was carried out only by bone marrow analysis because of 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 1–3 cases each. Prevalence of mixed- or pure-breed did not vary among the four 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 four 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 less than 10 years and 22 (34.9%) were more than 10 years. Graphical 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 less or more than 10 years did not vary among the four AL subgroups ($P = 0.085$).

Complete blood count (CBC) at diagnosis was available for 64 dogs, 61 (95.3%) had thrombocytopenia, and 58 (90.6%) had anaemia. Mean leucocyte count was $98.73 ± 110.72 \times 10^3 \, \mu L^{-1}$ (median, $60 \times 10^3 \, \mu L^{-1}$; range, $1.77–571.48 \times 10^3 \, \mu L^{-1}$): 45 (70.3%) dogs had leucocytosis and 9 (14.1%) had leucopenia; 50 (78.2%) had neutropenia and 2

Figure 1. Age distribution of 63 dogs diagnosed with AL.
had leukocytosis. Of 15 dogs (6.7%) had leukopenia and 14 (93.3%) had leukocytosis; finally, among AULs, 1 of 13 (76.5%) had leukocytosis; among AMLs, 8 of 25 (32%) had leukopenia and 5 (71.4%) had leukocytosis; among T-ALLs, 2 of 9 (22.2%) had leukopenia and 7 (77.8%) had leukocytosis; among B-ALLs, 2 of 17 (11.8%) dogs had WBC count within RI, 4 (16%) had leukopenia and 46 (71.9%) had lymphopenia; and 2 (3.1%) had neutrophilia; 46 (71.9%) had lymphopenia, and 2 (3.1%) lymphocytosis; and 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 four AL subgroups, except for WBC count abnormalities, that were significantly different among the four AL subgroups (P = 0.025). In particular, among B-ALLs, 2 of 17 (11.8%) dogs had WBC count within RI, 2 of 17 (11.8%) had leukopenia and 13 of 17 (76.5%) had leukocytosis; among T-ALLs, 2 of 7 (28.6%) had leukopenia and 5 (71.4%) had leukocytosis; among AMLs, 8 of 25 (32%) had leukopenia and 5 (71.4%) had leukocytosis; finally, among AULs, 1 of 15 dogs (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%) AUL cases. In particular, eight (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 six (46.2%) cases, haematological values were similar to those obtained on diagnosis: among them, three had been treated with corticosteroids alone, and three with a combination of corticosteroids and chemotherapy. In five (38.5%) dogs, haematological parameters improved: among them, one dog was treated with corticosteroids alone, subsequently relapsed when corticosteroids dosage was reduced, and died after 73 days; and four dogs received chemotherapy. Finally, in two (15.4%) cases, haematological values worsened after chemotherapy treatment.

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

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

When considering signalment, median ST (treated and untreated) was 8 days (range, 3–120 days) for pure-breed dogs (B-ALL, n = 3; T-ALL, n = 5; AML, n = 8; AUL, n = 6) and 15 days (range, 1–46 days) for mixed-breed dogs (B-ALL, n = 2; AML, n = 3; AUL, n = 2), 10 days (range, 4–120 days) for females (B-ALL, n = 3; T-ALL, n = 4; AML, n = 5; AUL, n = 4) and 7 days (range, 1–40 days) for males (B-ALL, n = 1; T-ALL, n = 1; AML, n = 6; AUL, n = 4), 7 days (range, 1–120 days) for dogs less than 10 years (B-ALL, n = 5; T-ALL, n = 4; AML, n = 7; AUL, n = 4) and 10 days (range, 7–90 days) for dogs more than 10 years (T-ALL, n = 1; AML, n = 4; AUL, n = 3).

When considering haematology results, median ST (treated and untreated) was 10 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 and 4–46 days, respectively); 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, $n = 6$); 1 and 5 days, respectively, for the two dogs with neutrophilia (AUL and B-ALL, respectively); 5 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, $n = 8$); 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, $n = 8$); 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 ST 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 days) for dogs receiving any chemotherapy protocol including cytosine arabinoside (B-ALL, $n = 1$; T-ALL, $n = 1$; AML, $n = 2$).

When rechecking 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 (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 neutrophilia 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. This 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 leukemias but no data on the clinical follow-up were reported.1 2

The study by Adam et al.1 included ALLs, AMLs and chronic lymphocytic leukemias (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 CD3) and against four different isoforms of CD11 (whereas we tested only CD11b). Similarly, Tasca et al.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 leukaemic lymphomas, whereas in this study and in the one by Adam et al., the final diagnosis was made
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, and associated with a short survival in dogs with neoplastic lymphocytosis. However, CD34 expression has been described in a subset of canine lymphoma, and CD34-negative ALs have also been reported. 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 this study overlap those reported in literature. 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 found a significant over-representation of Golden Retrievers in the ALL group compared with that in the control population. Age at diagnosis was similar among the three groups, and no significant difference in sex among AL subtypes 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 this study, but was not statistically significant. This discrepancy might be related to the inclusion in this 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 with 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 ageing. 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 with 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 due 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 with 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 leukaemias. 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.

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. Three of the four dogs treated with cytosine in this 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 this study achieved clinical and/or haematological remission. This is in agreement with what is reported in the veterinary literature. On the contrary, complete remission is achieved in up to 80% of cases in human medicine, depending on AL subtype, patient age at diagnosis and other prognostic factors. This difference could be due to a more aggressive behaviour of canine ALs compared with human ALs, or to a delay in the diagnosis. Further studies are needed to assess whether there is any dissimilarity in cytogenetic and molecular genetic abnormalities underlying neoplasia between canine and human ALs, which could further explain the different response to first treatment.

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

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

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