ZAP-70 expression is associated with increased risk of autoimmune cytopenias in CLL patients

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Autoimmune cytopenias (AIC) are frequent in chronic lymphocytic leukemia (CLL) patients, but risk factors and prognostic relevance of these events are controversial. Data about the influence on AIC of biological prognostic markers, as ZAP-70, are scanty. We retrospectively evaluated AIC in 290 CLL patients tested for ZAP-70 expression by immunohistochemistry on bone marrow biopsy at presentation. They were 185 men, median age 63 years, 77.9% Binet stage A, 17.6% B and 4.5% C. AIC occurred in 46 patients (16%): 31 autoimmune hemolytic anemias, 10 autoimmune thrombocytopenias, four Evans syndromes, and one pure red cell aplasia. Of the 46 cases of AIC, 37 (80%) occurred in ZAP-70 positive patients and nine (20%) in ZAP-70 negatives. ZAP-70 expression [Hazard Ratio (HR) = 7.42; 95% confidence interval (CI): 2.49–22.05] and age >65 years (HR = 5.41; 95% CI: 1.67–17.49) resulted independent risk factors for AIC. Among the 136 patients evaluated both for ZAP-70 expression and IGHV gene mutational status, the occurrence of AIC was higher in ZAP-70 positive/IGHV unmutated cases (35%) than in patients ZAP-70 negative/IGHV mutated (6%) or discordant for the two parameters (4%; P < 0.0001). In ZAP-70 positive patients, occurrence of AIC negatively influenced survival (HR = 1.75; 95% CI: 1.06–2.86). The high risk of developing AIC in ZAP-70 positive CLL, particularly when IGHV unmutated, should be considered in the clinical management. Am. J. Hematol. 85:494–498, 2010. © 2010 Wiley-Liss, Inc.

Introduction
Chronic lymphocytic leukemia (CLL) is known to be characterized by a profound dysregulation of the immune system, including autoimmune phenomena. The most frequent autoimmune complications in CLL are directed against blood cell antigens producing autoimmune cytopenias (AIC), namely autoimmune hemolytic anemia (AIHA), autoimmune thrombocytopenia (AITP) and, more rarely, pure red cell aplasia (PRCA). Of them, AIHA is the most common, with a reported prevalence in recent studies ranging from 3% to 10% [1–5]. AITP is estimated to be less frequent than AIHA in CLL, with an overall prevalence around 2%–5% [5–7]. About a third of AITP is associated with AIHA (Evans’ Syndrome) [8]. PRCA associated with CLL is quite rare, whereas the occurrence of immune neutropenia in CLL is disputed [8].

The prognostic relevance of AIHA occurrence in CLL patients is controversial (1–4,9), whereas AITP has been reported to be associated with inferior overall survival (OS) [6].

Older age, male sex, advanced stage, high lymphocyte count, and previous heavy treatment were reported to be associated to higher risk of AIHA, high lymphocyte count and positivity of direct antiglobulin test (DAT) with AITP [1,6,9,10]. The reported increased risk of AIHA after treatment with purine analogues has recently been questioned [3,9–12].

The correlation of CLL biological prognostic markers, as ZAP-70 expression and IGHV gene mutational status, with occurrence of AIC is uncertain, with the exception of the reported increased incidence of AITP in IGHV unmutated patients [6].

ZAP-70 protein expression in leukemic cells has been validated as reliable and independent marker capable of strongly influencing both progression-free survival and OS in CLL. Various techniques were used for testing ZAP-70 expression, including flow cytometry and immunohistochemistry (IHC) [13–17]. Although flow cytometry is commonly used, IHC was demonstrated to be a reliable method for this purpose [16–18].

To assess a possible correlation between ZAP-70 expression and other clinical and biological prognostic factors of CLL and the risk of AIHA, we analyzed our CLL patients tested for ZAP-70 expression by IHC on bone marrow (BM) biopsy at presentation.

Patients and Methods
Patients. We retrospectively evaluated 290 patients referring to the Hematology Department of Verona and diagnosed as CLL between June 1989 and December 2008, selected on the basis of the availability of ZAP-70 expression by leukemic cells tested by IHC on BM biopsy, performed within 6 months from diagnosis, and before any treatment.

Approval was obtained from the Azienda Ospedaliera di Verona institutional review board for this study.

Diagnosis of CLL was based or revised according to recent morphological and immunophenotypical criteria [19]. They were 185 men (64%) and 105 women, aged 33–85 years (median 63). At diagnosis, 226 patients were classified as Binet stage A (77.9%), 51 stage B (17.6%), and 13 stage C (4.5%). The median follow-up from diagnosis was 80 months (range 12–240 months). The finding at diagnosis of anemia or thrombocytopenia because of autoimmune phenomena was not considered as criteria for classifying patients as Binet stage C.

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Conflict of interest: Nothing to report.

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Determination of IGHV gene mutational status. IGHV mutational status was assessed in leukemic cells of 136 patients obtained from frozen or fresh peripheral blood (PB) samples collected at diagnosis after isolation by Ficoll gradient, as previously described [17]. The sequences with a germline homology \( \geq 98\% \) were considered unmutated, and those \(<98\% \) mutated [22].

Statistical analysis. The comparison of presenting clinical and laboratory features between the groups of patients with and without AIC was carried out with the chi-square test, Fisher exact test or Mann-Whitney test as appropriate. OS was defined as the time from diagnosis of CLL to occurrence of the first episode of AIC or last follow-up or death.

OS and time to AIC curves were estimated according to the Kaplan-Meier method. The association with prognostic factors was tested using the log-rank test. Univariate and multivariate analyses were performed using the Cox regression model, to obtain unadjusted and adjusted hazard ratios (HRs) and their 95% confidence intervals (95% CIs). All tests were bilateral at \( P < 0.05 \). The statistical analyses were performed using StatView (Abacus Concepts, Berkeley, CA).

Results

Patient's data

Fifty-six of 290 patients (16%) developed AIC. Clinical and laboratory characteristics of these patients are listed in Table I.

The most frequent form of AIC was AIHA because of warm autoantibodies (31 cases—11%), followed by AITP (10 cases—3%), Evans syndrome (4 cases—1%), and PRCA (1 case—0.3%). No cases of cold haemagglutinin diseases were recorded. The median age at occurrence of AIC was 67 years (range 41–85).

AIC occurred at diagnosis in 7 of 46 patients (15%) (four AIHA, two AITP, and one PRCA); in the remaining 39 patients, the median time from diagnosis to AIC was 52 months (range 4–147). AIHA and AITP developed after a median time from diagnosis of 58 (range 0–112) and 41 (range 0–147) months, respectively (Fisher exact test: \( P = ns \)). Four patients had alternated episodes of AIHA and AITP during the observation period.

The 7-year actuarial cumulative incidence of AIC was 17% and that of AIHA and AITP, 13% and 3%, respectively.

ZAP-70 evaluation by IHC on BM biopsies. Homogeneously cytoplasmic and/or nuclear ZAP-70 expression in neoplastic B lymphocytes was detected in 142 of 290 cases (49%).

Diagnostic criteria for AIC. AIHA was diagnosed in patients with a drop in Hb level below 10 gr/dl, associated with positive DAT for either IgG or complement fragment C3d and the presence of one or more indicators of hemolysis (increased indirect bilirubin and/or LDH, decreased haptoglobin, increased absolute reticulocyte count), with no other cause for anemia identified.

The diagnosis of AITP was made in patients with platelets \(< 100 \times 10^9\) with no evidence of hypersplenism, nor decreased BM platelet production (normal or increased megakaryocytes on BM examination) and no cytotoxic treatment in the last month [2,3].

The diagnosis of PRCA was based on Hb \(< 10 \text{ gr/dl}\), reticulocytopenia (\(<10 \times 10^9\text{/L}\)) and isolated absence of erythroid precursors in BM aspirate or biopsy. Patients developing different types of AIC during the disease course were classified according to the first episode occurred.

Treatment. Before the occurrence of AIC, 170 patients had been treated, of whom 71 with chlorambucil alone, 93 with fludarabine combined with cyclophosphamide (FC) and 66 with two or more lines of therapy (mostly chlorambucil followed by fludarabine or FC).

ZAP-70 expression was evaluated on BM biopsies taken at diagnosis. The method of IHC has been already published in detail [17]. The pattern of BM involvement was categorized according to Rozman et al. [20].

ZAP-70 and CD38 evaluation by flow cytometry. Evaluation of ZAP-70 and of CD38 by flow cytometry was performed in 181 and 209 patients, respectively. The method of flow cytometry has been already published in detail [17]. The cut-off value was set at 20% of ZAP-70 and at 7% of CD38 positive B-CLL cells, identified as CD19+/CD5+ cells [21].

ZAP-70 and CD38 evaluation by flow cytometry. ZAP-70 was expressed in 98% \(\pm 1\) of CD38 positive B-CLL cells in 92 of 181 tested cases (51%). ZAP-70 expression evaluated both by IHC and flow cytometry was concordant in 160 of 181 cases (88%) and, particularly, in 21 of 22 patients with AIC evaluated with both techniques.

CD38 was expressed in \(\geq 7\%\) of B-CLL cells in 114 of 209 tested cases (56%).

IGHV gene mutational status. In 69 of the 136 patients (51%) tested, the expressed IGHV gene showed 98% or greater identity with a known germ-line IGHV gene. IGHV gene mutational status was concordant with ZAP-70 expression evaluated by IHC in 110 of 136 patients (81%). Of the 26 discordant cases, 16 (12%) were IGHV mutated and ZAP-70 positive, 10 (7%) unmuted and ZAP-70 negative.

Association of AIC with clinical and biological prognostic parameters evaluated at diagnosis. Clinical features at diagnosis of patients who developed AIC compared with those of the remaining patients are detailed in Table I. Patients with AIC had significantly lower male/female ratio, higher PB lymphocyte count, and more frequent diffuse BM infiltration, unmutated IGHV genes, and expression of ZAP-70. No significant difference was found concerning Binet stage, age, and CD38 expression. Patients who developed AIHA and AITP, respectively, did not significantly differ according to the clinical and biological parameters evaluated at diagnosis (data not shown).

As shown in Table II, female sex, age \(> 65\) years, Binet stage B-C, PB lymphocyte count \(> 30 \times 10^9/\text{L}\), B2M levels \(> 2\) mg/dl, diffuse BM infiltration, ZAP-70 expression by IHC and unmutated IGHV status were associated with increased risk of developing AIC at univariate analysis. Only CD38 expression did not correlate with time to AIC.

 Associations with increased occurrence of AIC were confirmed by adjusted Cox model for ZAP-70 expression (HR = 7.42; 95% CI: 2.49–22.05) and age \(> 65\) years (HR = 4.62; 95% CI: 1.9–11.22). IGHV genes mutational status was not included in Cox model because of the fact that was evaluated only in 136 of 290 patients and because of its high concordance rate with ZAP-70 expression in patients who developed AIC (92%).

Thirty-seven of the 46 cases of AIC occurred in ZAP-70 positive patients (80%) and nine in ZAP-70 negatives.

### Table I. Characteristics at Diagnosis of 290 CLL Patients According to Occurrence of AIC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CLL without AIC (n = 244)</th>
<th>CLL with AIC (n = 46)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>163 (66.8%)</td>
<td>22 (47.8%)</td>
<td>0.018&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>2.01</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>Median age, yr (range)</td>
<td>62 (33–83)</td>
<td>66 (35–80)</td>
<td>0.235&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Binet stage</td>
<td>A</td>
<td>188 (77.5%)</td>
<td>31 (67.4%)</td>
</tr>
<tr>
<td>B</td>
<td>46 (18.8%)</td>
<td>11 (23.9%)</td>
<td>0.203&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>9 (3.7%)</td>
<td>4 (8.7%)</td>
<td></td>
</tr>
<tr>
<td>PB Lymphocyte, 10⁹/L</td>
<td>20.1 ± 1.6</td>
<td>27.6 ± 4.6</td>
<td>0.037&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Beta2-Microglobulin, mean ± SE mg/dl</td>
<td>1.97 ± 0.1</td>
<td>2.33 ± 0.3</td>
<td>0.256&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diffuse BM infiltration</td>
<td>77 (31.6%)</td>
<td>22 (47.8%)</td>
<td>0.041&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD38 positivity by flow cytometry ((\geq 7%) B cells)</td>
<td>94 (54.0%)</td>
<td>20 (57.1%)</td>
<td>0.862&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>ZAP-70 positivity by IHC</td>
<td>105 (43.0%)</td>
<td>37 (80.4%)</td>
<td>&lt;0.0001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
| IGHV unmutated (\(\leq 98\%\) homology) | 51 (45.9%) | 18 (72.0%) | 0.028<sup<f/sup>.

Statistically significant (P < 0.05) values are indicated in bold. AIIC, autoimmune cytopenia; PB, peripheral blood; BM, bone marrow; IHC, immunohistochemistry. |
TABLE II. Effect of Prognostic Factors on Time to AIC: Univariate and Multivariate Analysis (Hazard Ratio—95% CI)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted HR (95% CI)</th>
<th>Adjusted HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex</td>
<td>1.77 (1.00–3.16)</td>
<td>1.44 (0.62–3.31)</td>
</tr>
<tr>
<td>Age &gt; 65 years</td>
<td>2.08 (1.15–3.73)</td>
<td>4.62 (1.9–11.22)</td>
</tr>
<tr>
<td>Binet stage B–C</td>
<td>2.28 (1.19–4.35)</td>
<td>0.91 (0.31–2.71)</td>
</tr>
<tr>
<td>PB lymphocyte count &gt; 30 × 10⁹/L</td>
<td>2.34 (1.26–4.34)</td>
<td>2.62 (0.99–6.96)</td>
</tr>
<tr>
<td>Beta-2microglobulin &gt; 2 mg/dl</td>
<td>2.83 (1.23–6.48)</td>
<td>0.30 (0.48–2.14)</td>
</tr>
<tr>
<td>Diffuse BM infiltration</td>
<td>2.26 (1.21–4.10)</td>
<td>1.28 (0.50–3.25)</td>
</tr>
<tr>
<td>CD38 positivity by flow cytometry (≥7% B cells)</td>
<td>1.20 (0.61–2.34)</td>
<td>–</td>
</tr>
<tr>
<td>ZAP-70 expression by IHC</td>
<td>5.94 (2.83–12.47)</td>
<td>7.42 (2.49–22.05)</td>
</tr>
<tr>
<td>IGHV unmutated</td>
<td>3.80 (1.55–9.30)</td>
<td>–</td>
</tr>
<tr>
<td>(≥98% homology)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Statistically significant (P < 0.05) values are indicated in bold.
AIC, autoimmune cytopenia; PB, peripheral blood; BM, bone marrow; IHC, immunohistochemistry.

Values available in 209 patients; Values available in 136 patients.

TABLE III. Characteristics of Patients Who Developed AIC, According to ZAP-70 Expression

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ZAP-70 negative (9 pts)</th>
<th>ZAP-70 positive (37 pts)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female ratio</td>
<td>0.6</td>
<td>2.0</td>
<td>0.71*</td>
</tr>
<tr>
<td>Median age, yr (range)</td>
<td>61 (35–78)</td>
<td>66 (42–80)</td>
<td>0.57a</td>
</tr>
<tr>
<td>B–C Binet stage (%)</td>
<td>2 (22)</td>
<td>12 (32)</td>
<td>0.70a</td>
</tr>
<tr>
<td>CD38 positivity (%)</td>
<td>2 (22)</td>
<td>18 (65)</td>
<td>0.021*</td>
</tr>
<tr>
<td>IGHV unmutated (%)</td>
<td>0 (0)</td>
<td>18 (90)</td>
<td>–0.0001*</td>
</tr>
<tr>
<td>(≥98% homology) (%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Type of AIC (%)</td>
<td>6 (67)</td>
<td>25 (68)</td>
<td>0.59</td>
</tr>
<tr>
<td>AIHA</td>
<td>3 (33)</td>
<td>7 (19)</td>
<td>0.59</td>
</tr>
<tr>
<td>AITP</td>
<td>0</td>
<td>4 (11)</td>
<td>0.59</td>
</tr>
<tr>
<td>PRCA</td>
<td>0</td>
<td>1 (2)</td>
<td>0.59</td>
</tr>
<tr>
<td>Median follow-up, months (range)</td>
<td>136 (12–240)</td>
<td>91 (18–202)</td>
<td>0.22</td>
</tr>
<tr>
<td>Median time from diagnosis</td>
<td>70 (0–192)</td>
<td>41 (0–108)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

AIHA: autoimmune haemolytic anaemia; AITP: autoimmune thrombocytopenia; PRCA: pure red cell aplasia.

* Fisher’s exact test; aMann-Whitney; cchi-square test; Values available in 35 patients; Values available in 25 patients.

(20%). Characteristics of patients who developed AIC according to ZAP-70 expression were detailed in Table III. In particular, ZAP-70 negative cases did not differ from ZAP-70 positives with respect to age and stage at diagnosis, sex, and type of AIC. As expected, ZAP-70 positive patients were mostly CD38 positive and IGHV unmutated.

The 7-years projected cumulative incidence of AIC in ZAP-70 positive patients was 30% vs 4% of ZAP-70 negatives (P < 0.0001) (Fig. 1).

Of the 25 patients with AIC tested for IGHV mutational status, 18 (72%) resulted unmaturated and 7 (28%) mutated. Among the 136 patients tested for the mutational status, the 7-years occurrence of AIC in the 59 ZAP-70 positive/IGHV unmaturated cases was higher (35%) as compared with that of the 51 ZAP-70 negative/IGHV mutated patients (6%) and of the 26 patients discordant for the two prognostic parameters (4%) (P = 0.0004: Fig. 2). The two discordant patients who developed AIC were both ZAP-70 positive/IGHV mutated.

Association of AIC and treatment of CLL. The occurrence of AIC was not significantly different among untreated or treated patients and among those who received one or more lines of therapy.

AIC occurred in 28 treated patients: in detail, in 18 of 71 (25%) patients treated only with Chlorambucil, in 4 of 18 (22%) who received fludarabine alone and in 6 of 79 (8%) treated with FC (x² test: P = 0.011).

Overall Survival. We observed 87 disease- or treatment-related deaths (30%) and only three deaths in untreated patients for unrelated causes. The 10-years projected OS was 60%.

Among the clinical and biological markers that have been widely recognized as adverse prognostic factors in CLL patients at diagnosis, univariate analysis revealed age >65 years (P = 0.029), B-C Binet stage (P < 0.0001), PB lymphocyte count >30 × 10⁹/L (P = 0.010), B2MG levels >2 mg/dl (P < 0.0001), diffuse BM infiltration (P = 0.0011), ZAP-70 expression (P < 0.0001), CD38 positivity (P = 0.0004), and unmutated IGHV status (P < 0.0001), as adverse factors in terms of OS. As shown in Fig. 3, patients who experienced AIC during the observation period had a 10-year OS inferior (34%) than patients without AIC (67%; HR = 2.28; 95% CI: 1.46–3.56).

At multivariate analysis, AIC lost independent predictive power (HR = 1.21; 95% CI: 0.63–2.32) when computed in the Cox model with all clinical and biologic variables that resulted significant at univariate analysis. Only B-C Binet stage (HR = 2.57; 95% CI: 1.35–4.89), age >65 years (HR = 2.01; 95% CI: 0.92–3.72), and ZAP-70 expression (HR = 2.90; 95% CI: 1.47–5.73) maintained a significant impact on OS. When we stratified the patients according to ZAP-70 expression, occurrence of AIC demonstrated an adverse impact on survival only in ZAP-70 positive patients (HR = 1.75; 95% CI: 1.06–2.86) but not in ZAP-70 negative patients (Fig. 4).
Discussion

Hematological autoimmune complications are known to be frequent in CLL, being reported in up to 25% of patients during the disease course [7,8].

AIHA is recognized as the commonest hematological autoimmune complication in CLL [8]. The prevalence of AIHA we observed (11%) was higher as compared with some recent studies (1–3.5); this finding could be related not only to a possible selection of cases, but also to the long follow-up period of our patients. In fact, in our series AIHA occurred after a median time from diagnosis of 58 months. The prevalence of AITP (3%) we observed is comparable with that reported in the literature [6,8].

In our series, patients who developed AIHA had lower male/female ratio, higher PB lymphocytes count, more frequent diffuse BM infiltration as compared with patients without AIHA. Moreover, they had more frequently unmutated \( IGHV \) genes than patients with AITP. Other parameters reported as factors associated with AIHA as B2MG, advanced stage, high lymphocyte count [1,6,9,10,23] did not result significant when combined in the Cox model with ZAP-70 expression. Other factors associated with AIHA as B2MG, advanced stage, high lymphocyte count [1,6,9,10,23] did not result significant when combined in the Cox model with ZAP-70 expression. \( IGHV \) genes mutational status was not included in Cox model because of the fact that it was evaluated only in about half of patients.

ZAP-70 expression is usually tested on PB lymphocytes by flow cytometry, although this approach still presents standardization problems [24,25]. To assess ZAP-70 expression, we utilized immunohistochemical analysis on BM biopsies. The reliability of this technique and the close correlation between ZAP-70 as detected by IHC in BM biopsies and PFS and OS in CLL patients have been previously demonstrated [17,18]. Moreover, this IHC method of evaluation of ZAP-70 using formalin fixed BM biopsies was as sensitive as flow cytometry to detect ZAP-70 expression [26]. The concordance rate of ZAP-70 expression tested by IHC and flow cytometry, respectively, was concordant in 89% of cases. The possibility of discordance was more frequent in patients treated only with CLB or fludarabine and other purine analogs has been extensively reported [11,12]. In our series, treatment did not seem to increase significantly the risk of AIC development. Nevertheless, consisting with previous observations [3,10], AIC was more frequent in patients treated only with CLB or FLU alone as compared with those receiving FC.

The impact of treatment and in particular of purine analogs on AIHA occurrence in CLL patients is controversial. An increased risk of severe AIHA in CLL patients receiving fludarabine and other purine analogs has been extensively reported [11,12]. In our series, treatment did not seem to increase significantly the risk of AIC development. Nevertheless, consisting with previous observations [3,10], AIC was more frequent in patients treated only with CLB or FLU alone as compared with those receiving FC.

The impact of AIHA and generally of AIC on CLL prognosis is controversial. The majority of reports [1,2,5] found that AIHA had no independent effect on OS. However, Dearden et al. [4], recently reported inferior survival for patients developing AIHA or with positive DAT on a large prospective series. Also, AITP was recently reported to have an adverse influence on survival in patients with CLL.
Our data are consistent with a negative impact of AIC on survival of patients with CLL, although only at univariate analysis. Interestingly, this adverse impact on survival seemed limited to ZAP-70 positive patients, suggesting that the occurrence of AIC could worsen the unfavourable influence of this prognostic marker. The adverse influence of AIC on survival could be related to the need of immunosuppressive therapy and, consequently, to the higher risk of infectious complications.

In conclusion, we confirm that AIC represent a common complication of CLL during the disease course, and we show that their occurrence is associated with adverse biological prognostic factors, occurring in older patients and in patients with ZAP-70 positive expression and unmutated IGHV genes. In this subset of CLL patients, the high risk of AIC should be taken into account in the clinical management and treatment planning.

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References