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ZAP-70 EXPRESSION IS ASSOCIATED WITH INCREASED RISK OF AUTOIMMUNE CYTOPENIAS IN CLL PATIENTS

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ABSTRACT

Autoimmune cytopenias (AIC) are frequent in 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 males, median age 63 years, 77.9% Binet stage A, 17.6% B and 4.5% C.

AIC occurred in 46 patients (16%): 31 autoimmune haemolytic anaemias, 10 autoimmune thrombocytopenias, 4 Evans syndromes and 1 pure red cell aplasia. Of the 46 cases of AIC, 37 (80%) occurred in ZAP-70positive patients and 9 (20%) in ZAP-70negatives. ZAP-70 expression (HR=7.42; 95%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 for ZAP-70 expression and *IGHV* status the occurrence of AIC was higher in ZAP-70positive/*IGHV*unmutated cases (35%) than in patients ZAP-70negative/*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-70positive CLL, particularly when *IGHV*unmutated, should be considered in the clinical management.

INTRODUCTION

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 haemolytic anaemia (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 anti-globulin test (DAT) with AITP (1,6,9,10). The reported elevated risk of AIHA following 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 employed, IHC was demonstrated to be a reliable method for this purpose (16-18).

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3 In order to assess a possible correlation between ZAP-70 expression and other clinical and
4 biological prognostic factors of CLL and the risk of AIC, we analyzed our CLL patients tested for
5 ZAP-70 expression by IHC on bone marrow (BM) biopsy at presentation.
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10 11 12 **PATIENTS AND METHODS**

13 14 15 *Patients*

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18 We retrospectively evaluated 290 patients referring to the Haematology Department of
19 Verona and diagnosed as CLL between June 1989 and December 2008, selected on the basis of the
20 availability of ZAP-70 expression by leukemic cells tested by IHC on BM biopsy, performed within
21 6 months from diagnosis, and before any treatment.
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28 Approval was obtained from the Azienda Ospedaliera of Verona institutional review board for
29 this study.
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33 Diagnosis of CLL was based or revised according to recent morphological and
34 immunophenotypical criteria (19). They were 185 males (64%) and 105 females, aged 33 to 85
35 years (median 63). At diagnosis 226 patients were classified as Binet stage A (78%), 51 stage B
36 (18%) and 13 stage C (4%). The median follow-up from diagnosis was 80 months (range 12–240
37 months). The finding at diagnosis of anaemia or thrombocytopenia due to autoimmune phenomena
38 was not considered as criteria for classifying patients as Binet stage C.
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47 48 *Diagnostic criteria for AIC*

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50 AIHA was diagnosed in patients with a drop in Hb level below 10 gr/dl, associated with
51 positive DAT for either IgG or complement fragment C3d and the presence of one or more
52 indicators of haemolysis (increased indirect bilirubin and/or LDH, decreased haptoglobin, increased
53 absolute reticulocyte count), with no other cause for anaemia identified.
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59 The diagnosis of AITP was made in patients with platelets $<100 \times 10^9$ with no evidence of
60 hypersplenism, nor decreased BM platelet production (normal or increased megacariocytes on BM

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3 examination) and no cytotoxic treatment in the last month (2, 5).

4
5 The diagnosis of PRCA was based on Hb < 10 gr/dl, reticulocytopenia (< 10x10⁹/L) and
6
7 isolated absence of erythroid precursors in BM aspirate or biopsy.

8
9 Patients developing different types of AIC during the disease course were classified according
10
11 to the first episode occurred.

12 13 14 **Treatment**

15
16 Before the occurrence of AIC 170 patients had been treated, of whom 71 with Chlorambucil
17
18 alone, 33 with Fludarabine combined with Cyclophosphamide (FC) and 66 with 2 or more lines of
19
20 therapy (mostly Chlorambucil followed by Fludarabine or FC).

21 22 23 **ZAP-70 evaluation by IHC**

24
25 In all patients ZAP-70 expression was evaluated on BM biopsies taken at diagnosis. The
26
27 method of IHC has been already published in detail (17). The pattern of BM involvement was
28
29 categorized according to Rozman *et al*, 1984 (20).

30 31 32 **ZAP-70 and CD38 evaluation by flow cytometry**

33
34 Evaluation of ZAP-70 and of CD38 by flow cytometry was performed in 181 and 209
35
36 patients, respectively. The method of flow cytometry has been already published in detail (17). The
37
38 cut-off value was set at 20% of ZAP-70 and at 7% of CD38 positive B-CLL cells, identified as
39
40 CD19+/CD5+ cells (21).

41 42 43 **Determination of IGHV gene mutational status**

44
45 *IGHV* mutational status was assessed in leukemic cells of 136 patients obtained from frozen
46
47 or fresh peripheral blood (PB) samples collected at diagnosis after isolation by Ficoll gradient, as
48
49 previously described (17). The sequences with a germline homology $\geq 98\%$ were considered
50
51 unmutated, and those <98% mutated (22).

52 53 54 **Statistical analysis**

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3 The comparison of presenting clinical and laboratory features between the groups of patients
4 with and without AIC was carried out with the χ^2 test, Fisher exact test or Mann-Whitney test as
5 appropriate. OS was defined as the time from diagnosis of CLL to death or last follow-up. Time to
6 AIC was defined as the time from diagnosis of CLL to occurrence of the first episode of AIC or last
7 follow-up or death.
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11 OS and time to AIC curves were estimated according to the Kaplan-Meier method. The
12 association with prognostic factors was tested using the log-rank test. Univariate and multivariate
13 analyses were performed using the Cox regression model, in order to obtain unadjusted and
14 adjusted Hazard Ratios (HR) and their 95% confidence intervals (95% CI). All tests were bilateral
15 at $p < 0.05$. The statistical analyses were performed using StatView (Abacus Concepts, Berkeley,
16 CA).
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30 RESULTS

31 Patient's data

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35 Forty-six out of 290 patients (16%) developed AIC. Clinical and laboratory characteristics of
36 these patients are listed in Table 1.
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40 The most frequent form of AIC was AIHA due to warm autoantibodies (31 cases – 11%),
41 followed by AITP (10 cases – 3%), Evans Syndrome (4 cases - 1%) and PRCA (1 case - 0.3%). No
42 cases of cold haemagglutinin diseases was recorded. The median age at occurrence of AIC was 67
43 years (range 41-85).
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49
50 AIC occurred at diagnosis in 7/46 patients (15%) (4 AIHA, 2 AITP and 1 PRCA); in the
51 remaining 39 patients the median time from diagnosis to AIC was 52 months (range 4–147). AIHA
52 and AITP developed after a median time from diagnosis of 58 (range 0-112) and 41 (range 0-147)
53 months respectively (Fisher exact test: $P = ns$). Four patients had alternated episodes of AIHA and
54 AITP during the observation period.
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The 7-year actuarial cumulative incidence of AIC was 17% and that of AIHA and AITP 13%

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2
3 and 3%, respectively.
4

5 ***ZAP-70 evaluation by IHC on BM biopsies***

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8 Homogeneous cytoplasmic and/or nuclear ZAP-70 expression in neoplastic B lymphocytes
9
10 was detected in 142/290 cases (49%).
11

12 ***ZAP-70 and CD38 evaluation by flow cytometry***

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14
15 ZAP-70 was expressed in $\geq 20\%$ of B-CLL cells in 92/181 tested cases (51%). ZAP-70
16
17 expression evaluated both by IHC and flow cytometry was concordant in 160/181 cases (88%) and,
18
19 particularly, in 21/22 patients with AIC evaluated with both techniques.
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21
22 CD38 was expressed in $\geq 7\%$ of B-CLL cells in 114/209 tested cases (56%).
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24 ***IGHV gene mutational status***

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26
27 In 69 of the 136 patients (51%) tested, the expressed *IGHV* gene showed 98% or greater
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29 identity with a known germ-line *IGHV* gene. *IGHV* gene mutational status was concordant with
30
31 ZAP-70 expression evaluated by IHC in 110/136 patients (81%). Of the 26 discordant cases, 16
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33 (12%) were *IGHV* mutated and ZAP-70 positive, 10 (7%) unmutated and ZAP-70 negative.
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36 ***Association of AIC with clinical and biological prognostic parameters evaluated at diagnosis***

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39 Clinical features at diagnosis of patients who developed AIC compared to those of the
40
41 remaining patients are detailed in Table 1. Patients with AIC had significantly lower male/female
42
43 ratio, higher PB lymphocyte count and more frequent diffuse BM infiltration, unmutated *IGHV*
44
45 genes and expression of ZAP-70. No significant difference was found concerning Binet stage, age
46
47 and CD38 expression. Patients who developed AIHA and AITP respectively did not significantly
48
49 differ according to the clinical and biological parameters evaluated at diagnosis (data not shown).
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53 As shown in Table 2, female sex, age >65 years, Binet stage B-C, PB lymphocyte count \geq
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55 $30 \times 10^9/L$, B2MG levels >2mg/dl, diffuse BM infiltration, ZAP-70 expression by IHC and
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57 unmutated *IGHV* status were associated with increased risk of developing AIC at univariate
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59 analysis. Only CD38 expression did not correlate with time to AIC.
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3 Associations with increased occurrence of AIC were confirmed by adjusted Cox model for
4 ZAP-70 expression (HR 7.42; 95% CI: 2.49-22.05) and age >65 years (HR 4.62; 95% CI: 1.9-
5 11.22). *IGHV* genes mutational status was not included in Cox model due to the fact that was
6 evaluated only in 136/290 patients and due to its high concordance rate with ZAP-70 expression in
7 patients who developed AIC (92%).
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15 Thirty-seven out of the 46 cases of AIC occurred in ZAP-70 positive patients (80%) and 9 in
16 ZAP-70 negatives (20%). Characteristics of patients who developed AIC according to ZAP-70
17 expression were detailed in Table 3. In particular, ZAP-70 negative cases did not differ from ZAP-
18 70 positives with respect to age and stage at diagnosis, sex, type of AIC. As expected, ZAP-70
19 positive patients were mostly CD38 positive and *IGHV* unmutated.
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27 The 7-years projected cumulative incidence of AIC in ZAP-70 positive patients was 30% vs
28 4% of ZAP-70 negatives ($P < 0.0001$) (Fig.1).
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32 Of the 25 patients with AIC tested for *IGHV* mutational status, 18 (72%) resulted unmutated,
33 7 (28%) mutated. Among the 136 patients tested for the mutational status the 7-years occurrence of
34 AIC in the 59 ZAP-70positive/*IGHV*unmutated cases was higher (35%) as compared to that of the
35 51 ZAP-70negative/*IGHV*mutated patients (6%) and of the 26 patients discordant for the two
36 prognostic parameters (4%) ($P = 0.0004$) (Fig.2). The 2 discordant patients who developed AIC
37 were both ZAP-70positive/*IGHV*mutated.
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45 46 ***Association of AIC and treatment of CLL***

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48 The occurrence of AIC was not significantly different among untreated or treated patients and
49 among those who received one or more lines of therapy.
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53 AIC occurred in 28 treated patients: in detail, in 18/71 (25%) patients treated only with
54 Chlorambucil, in 4/18 (22%) who received Fludarabine alone and in 6/79 (8%) treated with FC (χ^2
55 test: $P = 0.011$).
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60 ***Overall Survival***

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3 We observed 87 disease or treatment related deaths (30%) and only 3 deaths in untreated
4 patients for unrelated causes. The 10-years projected OS was 60%.
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8 Among the clinical and biological markers that have been widely recognized as adverse
9 prognostic factors in CLL patients at diagnosis, univariate analysis revealed age >65 years
10 ($P=0.029$), B-C Binet stage ($P<0.0001$), PB lymphocyte count $\geq 30 \times 10^9/L$ ($P=0.010$), B2MG levels
11 >2mg/dl ($P<0.0001$), diffuse BM infiltration ($P=0.0011$), ZAP-70 expression ($P<0.0001$), CD38
12 positivity ($P=0.0004$) and unmutated *IGHV* status ($P<0.0001$), as adverse factors in terms of OS. As
13 shown in Figure 3, patients who experienced AIC during the observation period had a 10-years OS
14 inferior (34%) than patients without AIC (67%) (HR=2.28; 95%CI: 1.46-3.56).
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25 At multivariate analysis, AIC lost independent predictive power (HR=1.21; 95% CI: 0.63-
26 2.32) when computed in the Cox model with all clinical and biologic variables that resulted
27 significant at univariate analysis. Only B-C Binet stage (HR=2.57; 95% CI: 1.35-4.89), age >65
28 years (HR=2.01; 95% CI: 0.92-3.72) and ZAP-70 expression (HR=2.90; 95% CI: 1.47-5.73)
29 maintained a significant impact on OS. When we stratified the patients according to ZAP-70
30 expression, occurrence of AIC demonstrated an adverse impact on survival only in ZAP-70 positive
31 patients (HR=1.75; 95% CI: 1.06-2.86) but not in ZAP-70 negative patients (fig 4).
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41 DISCUSSION

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43 Haematological autoimmune complications are known to be frequent in CLL, being reported
44 in up to 25% of patients during the disease course (7, 8).
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49 AIHA is recognized as the commonest haematological autoimmune complication in CLL (8).
50 The prevalence of AIHA we observed (11%) was higher as compared to some recent studies (1-
51 3,5); this finding could be related in part to a possible selection of cases, but also to the long follow
52 up period of our patients. In fact, in our series AIHA occurred after a median time from diagnosis of
53 58 months. The prevalence of AITP (3%) we observed is comparable with that reported in the
54 literature (6,8).
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3 In our series patients who developed AIC had lower male/female ratio, higher PB
4 lymphocytes count, more frequent diffuse BM infiltration as compared to patients without AIC.
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6 Moreover they had more frequently unmutated *IGHV* genes and positive expression of ZAP-70. At
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8 multivariate analysis time to AIC was significantly correlated only with age >65 years and ZAP-70
9
10 expression. Other parameters reported as factors associated with AIC as B2MG, advanced stage,
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12 high lymphocyte count (1,6,9,10,23) did not result significant when combined in the Cox model
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14 with ZAP-70 expression. *IGHV* genes mutational status was not included in Cox model due to the
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16 fact that it was evaluated only in about half of patients.
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22 ZAP-70 expression is usually tested on PB lymphocytes by flow cytometry, although this
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24 approach still presents standardization problems (24,25). To assess ZAP-70 expression we utilized
25
26 immunohistochemical analysis on BM biopsies. The reliability of this technique and the close
27
28 correlation between ZAP-70 as detected by IHC in BM biopsies and PFS and OS in CLL patients
29
30 have been previously demonstrated (17,18). Moreover this IHC method of evaluation of ZAP-70
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32 using formalin fixed BM biopsies taken at diagnosis allowed the retrospective evaluation of patients
33
34 with long follow-up. In our series ZAP-70 expression tested by IHC and flow cytometry
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36 respectively was concordant in 89% of cases. The possibility of discordance between the two
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38 methods probably did not influence our results: in fact in all but one of the 22 patients with AIC,
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40 analyzed with both techniques, ZAP-70 expression results were concordant. The concordance rate of
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42 ZAP-70 expression evaluated by IHC and *IGHV* mutational status we observed (81%) was
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44 comparable with reported data, which range between 77% to 92%, depending on the different series
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46 and techniques (13-15,17,26).
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53 We found ZAP-70 expression evaluated by IHC in 80% of patients with AIC; similar results
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55 are so far reported only in a small series of 5 AIHA (5). The 7-years cumulative incidence of AIC in
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57 ZAP-70 positive patients was 30% vs 4% of ZAP-70 negatives ($P<0.0001$).
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60 In our series an *IGHV* genes unmutated status was documented in 72% of patient with AIC, in
keeping with a previous report of 13/20 CLL patients with AIHA (65%) and 15/20 patients (75%)

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3 with AITP (5,27) Moreover, with the limit of the fact that evaluation was performed only in about
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5 50% of cases, CLL patients with both ZAP-70 expression and unmutated *IGHV* genes presented a
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7 incidence of AIC at 7-years (35%) significantly superior to that of ZAP-70 negative patients with
8
9 mutated genes (6%) and of patients discordant for the two prognostic parameters (4%) ($P < 0.0004$).
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11

12 The pathogenesis of AIC in CLL is still unclear. These autoimmune haematological
13
14 phenomena are usually caused by polyclonal IgG autoantibodies produced by non-malignant B-
15
16 lymphocytes and different from the Ig secreted by leukemic cells. In CLL, AIC are probably linked
17
18 to the profound immune dysfunction that characterizes this disease, particularly in advanced phases.
19
20 It has been hypothesized that autoimmune phenomena could be a consequence of a defect of
21
22 regulatory T cells. Leukemic B cells might contribute to autoimmune complications through
23
24 aberrant antigenic presentation (28). Since AIHA is reported to be more frequent in advanced CLL,
25
26 an increased risk of AIHA (and generally of AIC) in patients with ZAP-70 expression could be
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28 related to a more aggressive disease behaviour and more profound immune defect. In CLL different
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30 types of AIC are probably strictly correlated. In fact in our series risk factors for both AIHA and
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32 AITP were similar and clinical manifestations not infrequently overlapping as in Evans Syndrome
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34 or in cases of AITP occurring after AIHA or viceversa.
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41 The impact of treatment and in particular of purine analogs on AIHA occurrence in CLL
42
43 patients is controversial. An increased risk of severe AIHA in CLL patients receiving fludarabine
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45 and other purine analogs has been extensively reported (11,12). In our series treatment did not
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47 appear to increase significantly the risk of AIC development. Nevertheless, consisting with previous
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49 observations (3,10), AIC was more frequent in patients treated only with CLB or FLU alone as
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51 compared to those receiving FC.
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54 The impact of AIHA and generally of AIC on CLL prognosis is controversial. The majority of
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56 reports (1,2,5) found that AIHA had no independent effect on OS. However, Dearden et al (4),
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58 recently reported inferior survival for patients developing AIHA or with positive DAT on a large
59
60 prospective series. Also AITP was recently reported to have an adverse influence on survival in

1
2
3 patients with CLL (6). Our data are consistent with a negative impact of AIC on survival of patients
4
5 with CLL, although only at univariate analysis. Interestingly, this adverse impact on survival
6
7 appeared limited to ZAP-70 positive patients, suggesting that the occurrence of AIC could worsen
8
9 the unfavourable influence of this prognostic marker. The adverse influence of AIC on survival
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11 could be related to the need of immunosuppressive therapy and, consequently, to the higher risk of
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13 infectious complications.
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16
17 In conclusion, we confirm that AIC represent a common complication of CLL during the
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19 disease course, and we show that their occurrence is associated with adverse biological prognostic
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21 factors, occurring in older patients and in patients with ZAP-70 positive expression and unmutated
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23 *IGHV* genes. In this subset of CLL patients the high risk of AIC should be taken into account in the
24
25 clinical management and treatment planning.
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27

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REFERENCES

- 1 - Mauro FR, Foa R, Cerretti R et al. Autoimmune hemolytic anemia in chronic lymphocytic leukemia: clinical, therapeutic, and prognostic features. *Blood*. 2000;95: 2786-2792.
- 2 - Kyasa MJ, Parrish RS, Schichman SA, Zent CS. Autoimmune cytopenia does not predict poor prognosis in chronic lymphocytic leukemia/small lymphocytic lymphoma. *Am J Hematol*. 2003;74: 1-8.
- 3 - Borthakur G, O'Brien S, Wierda WG et al. Immune anaemias in patients with chronic lymphocytic leukaemia treated with fludarabine, cyclophosphamide and rituximab--incidence and predictors. *Br J Haematol*. 2007;136: 800-805.
- 4 - Dearden C, Wade R, Else M et al; UK National Cancer Research Institute (NCRI); Haematological Oncology Clinical Studies Group; NCRI CLL Working Group. The prognostic significance of a positive direct antiglobulin test in chronic lymphocytic leukemia: a beneficial effect of the combination of fludarabine and cyclophosphamide on the incidence of hemolytic anemia. *Blood*. 2008;111: 1820-1826
- 5 - Zent CS, Ding W, Schwager SM et al. The prognostic significance of cytopenia in chronic lymphocytic leukaemia/small lymphocytic lymphoma. *Br J Haematol*. 2008;141: 615-621
- 6 - Visco C, Ruggeri M, Evangelista LM et al. Impact of immune thrombocytopenia on the clinical course of chronic lymphocytic leukemia. *Blood*. 2008;111: 1110-1116.
- 7 - Dearden C. Disease-specific complications of chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program*. 2008: 450-456.
- 8 - Hamblin TJ. Autoimmune complications of chronic lymphocytic leukemia. *Semin Oncol*. 2006;33: 230-239.
- 9- Barcellini W, Capalbo S, Agostinelli RM, et al; GIMEMA Chronic Lymphocytic Leukemia Group. Relationship between autoimmune phenomena and disease stage and therapy in B-cell chronic lymphocytic leukemia. *Haematologica*. 2006;91: 1689-92.

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2
3 10- Catovsky D, Richards S, Matutes E et al.; UK National Cancer Research Institute (NCRI)
4
5 Haematological Oncology Clinical Studies Group; NCRI Chronic Lymphocytic Leukaemia
6
7 Working Group. Assessment of fludarabine plus cyclophosphamide for patients with chronic
8
9 lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial. *Lancet*. 2007;370:
10
11 230-239
12
13
14
15 11- Di Raimondo F, Giustolisi R, Cacciola E et al. Autoimmune hemolytic anemia in chronic
16
17 lymphocytic leukemia patients treated with fludarabine. *Leuk Lymphoma*. 1993;11: 63-68.
18
19
20 12- Weiss RB, Freiman J, Kweder SL et al. Haemolytic anaemia after fludarabine therapy for
21
22 chronic lymphocytic leukemia. *J Clin Oncol*. 1998;16: 1885-1889.
23
24
25 13- Crespo M, Bosch F, Villamor N et al. ZAP-70 expression as a surrogate for immunoglobulin-
26
27 variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med*. 2003;348: 1764-
28
29 1775.
30
31
32 14- Orchard JA, Ibbotson RE, Davis Z et al. ZAP-70 expression and prognosis in chronic
33
34 lymphocytic leukaemia. *Lancet*. 2004;363: 105-111.
35
36
37 15- Rassenti LZ, Huynh L, Toy TL et al. ZAP-70 compared with immunoglobulin heavy-chain
38
39 gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N*
40
41 *Engl J Med*. 2004;351: 893-901.
42
43
44 16- Admirand JH, Rassidakis GZ, Abruzzo LV et al. Immunohistochemical detection of ZAP-70 in
45
46 341 cases of non-Hodgkin and Hodgkin lymphoma. *Modern Pathology*. 2004;17: 954-961.
47
48
49 17- Zanotti R, Ambrosetti A, Lestani M et al. ZAP-70 expression, as detected by
50
51 immunohistochemistry on bone marrow biopsies from early-phase CLL patients, is a strong
52
53 adverse prognostic factor. *Leukemia*. 2007;21: 102-109.
54
55
56 18- Sabattini E, Orduz R, Campidelli C et al. B cell chronic lymphocytic leukaemia/small
57
58 lymphocytic lymphoma: role of ZAP70 determination on bone marrow biopsy specimens.
59
60 *Journal of Clinical Pathology*. 2007;60: 627-32.

- 1
2
3 19- Hallek M, Cheson BD, Catovsky D et al; International Workshop on Chronic Lymphocytic
4
5 Leukemia. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report
6
7 from the International Workshop on Chronic Lymphocytic Leukemia updating the National
8
9 Cancer Institute-Working Group 1996 guidelines. *Blood*. 2008;111: 5446-5456.
10
11
12 20- Rozman C, Montserrat E, Rodríguez-Fernández JM et al. Bone marrow histologic pattern - the
13
14 best single prognostic parameter in chronic lymphocytic leukemia: a multivariate survival
15
16 analysis of 329 cases. *Blood*. 1984;64: 642-648.
17
18
19 21- Ghia P, Guida G, Stella S, Gottardi D et al. The pattern of CD38 expression defines a distinct
20
21 subset of chronic lymphocytic leukemia (CLL) patients at risk of disease progression. *Blood*.
22
23 2003;101:1262-1269.
24
25
26 22- Hamblin TJ, Davis Z, Gardiner A et al. Unmutated Ig V(H) genes are associated with a more
27
28 aggressive form of chronic lymphocytic leukemia. *Blood*. 1999;94: 1848-1854.
29
30
31 23- Duek A, Shvidel L, Braester A, Berrebi A. Clinical and immunologic aspects of B chronic
32
33 lymphocytic leukemia associated with autoimmune disorders. *Isr Med Assoc J*. 2006;8: 828
34
35
36 24- Letestu R, Rawstron A, Ghia P et al. Evaluation of ZAP-70 expression by flow cytometry in
37
38 chronic lymphocytic leukemia: A multicentric international harmonization process. *Cytometry*
39
40 *B Clin Cytom*. 2006;70: 309-314.
41
42
43 25 Gachard N, Salviat A, Boutet C et al; GEIL Multicenter study of ZAP-70 expression in patients
44
45 with B-cell chronic lymphocytic leukemia using an optimized flow cytometry method.
46
47 *Haematologica*. 2008;93: 215-223.
48
49
50 26- Rassenti LZ, Jain S, Keating MJ et al. Relative value of ZAP-70, CD38, and immunoglobulin
51
52 mutation status in predicting aggressive disease in chronic lymphocytic leukemia. *Blood*.
53
54 2008;112: 1923-1930.
55
56
57 27- Visco C, Giaretta I, Ruggeri M et al. Un-mutated IgVH in chronic lymphocytic leukemia is
58
59 associated with a higher risk of immune thrombocytopenia. *Leukemia*. 2007;21: 1092-1093.
60

1
2
3 28- Hall AM, Vickers MA, McLeod E, Barker RN. Rh autoantigen presentation to helper T cells in
4
5 chronic lymphocytic leukaemia by malignant B cells. Blood. 2005;105: 2007-2015.
6
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LEGENDS

Figure 1: Time to autoimmune cytopenia from CLL diagnosis according to ZAP-70 expression (290 patients)

Figure 2: Time to autoimmune cytopenia from CLL diagnosis according to ZAP-70 expression and IGHV genes mutational status (136 patients)

Figure 3: Overall Survival according to the occurrence of autoimmune cytopenia (290 patients).

Figure 4: Overall Survival according to the occurrence of autoimmune cytopenia and ZAP-70 expression (290 patients).

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Table 1. Characteristics at diagnosis of 290 CLL patients according to occurrence of AIC

<i>parameters</i>	<i>CLL without AIC</i> (<i>n=244</i>)	<i>CLL with AIC</i> (<i>n=46</i>)	<i>P</i>
male sex	163 (66.8%)	22 (47.8%)	0.018*
male/female ratio	2.01	0.92	
median age, yr (range)	62 (33-83)	66 (35-80)	0.235#
Binet stage			
A	189 (77.5%)	31 (67.4%)	
B	46 (18.8%)	11 (23.9%)	0.203§
C	9 (3.7%)	4 (8.7%)	
PB Lymphocyte, mean±SE x10 ⁹ /L	20.1 ± 1.6	27.6 ± 4.6	0.037#
Beta2microglobulin, mean±SE mg/dl	1.97 ± 0.1	2.33 ± 0.3	0.258#
Diffuse BM infiltration	77 (31.6%)	22 (47.8%)	0.041*
CD38 positivity by flow cytometry (≥7% B cells) [°]	94 (54.0%)	20 (57.1%)	0.852*
ZAP-70 positivity by IHC	105 (43.0%)	37 (80.4%)	<0.0001*
<i>IGHV</i> unmutated (≥ 98% homology) ^{°°}	51 (45.9%)	18 (72.0%)	0.026*

Statistically significant ($P < 0.05$) values are indicated in bold

*Fisher's exact test.

#Mann-Witney.

§ chi-square test.

[°] Values available in 209 patients, ^{°°} values available in 136 patients

AIC, autoimmune cytopenia; PB, peripheral blood; BM, bone marrow, IHC, immunohistochemistry

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60**Table 2.** Effect of prognostic factors on time to AIC: univariate and multivariate analysis (Hazard Ratio - 95% CI)

<i>Variable</i>	<i>Unadjusted HR (95% CI)</i>	<i>Adjusted HR (95% CI)</i>
female sex	1.77 (1.00-3.16)	1.44 (0.62-3.31)
age >65 years	2.08 (1.15-3.73)	4.62 (1.9-11.22)
Binet stage B-C	2.28 (1.19-4.35)	0.91 (0.31-2.71)
PB lymphocyte count $\geq 30 \times 10^9/L$	2.34 (1.26-4.34)	2.62 (0.99-6.96)
Beta2microglobulin > 2 mg/dl	2.83 (1.23-6.48)	0.30 (0.48-2.14)
Diffuse BM infiltration	2.26 (1.21-4.10)	1.28 (0.50-3.25)
CD38 positivity by flow cytometry ($\geq 7\%$ B cells) [°]	1.20 (0.61-2.34)	-
ZAP-70 expression by IHC	5.94 (2.83-12.47)	7.42 (2.49-22.05)
<i>IGHV</i> unmutated ($\geq 98\%$ homology) ^{°°}	3.80 (1.55-9.30)	-

Statistically significant ($P < 0.05$) values are indicated in bold[°] Values available in 209 patients, ^{°°} values available in 136 patients

AIC, autoimmune cytopenia; PB, peripheral blood; BM, bone marrow, IHC, immunohistochemistry

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Table 3. Characteristics of patients who developed AIC, according to ZAP-70 expression

<i>Parameters</i>	<i>ZAP-70 neg 9 pts</i>	<i>ZAP-70 pos 37 pts</i>	<i>P</i>
male/female ratio	0.8	1.2	0.71*
median age, yr (range)	61 (35-78)	66 (42-80)	0.57#
B-C Binet stage (%)	2 (22)	12 (32)	0.70*
CD38 positivity by flow-cytometry ($\geq 7\%$ B cells) [°] (%)	2 (22)	18 (69)	0.021*
<i>IGHV</i> unmutated ($\geq 98\%$ homology) ^{°°} (%)	0 (0)	18 (90)	<0.0001*
Type of AIC (%)			
- AIHA	6 (67)	25 (68)	
- AITP	3 (33)	7 (19)	0.59§
- Evans syndrome	0	4 (11)	
- PRCA	0	1 (2)	
Median follow-up, months (range)	136 (12-240)	91 (18-202)	0.22#
Median time from diagnosis to occurrence of AIC, months (range)	75 (0-192)	41 (0-108)	0.31#

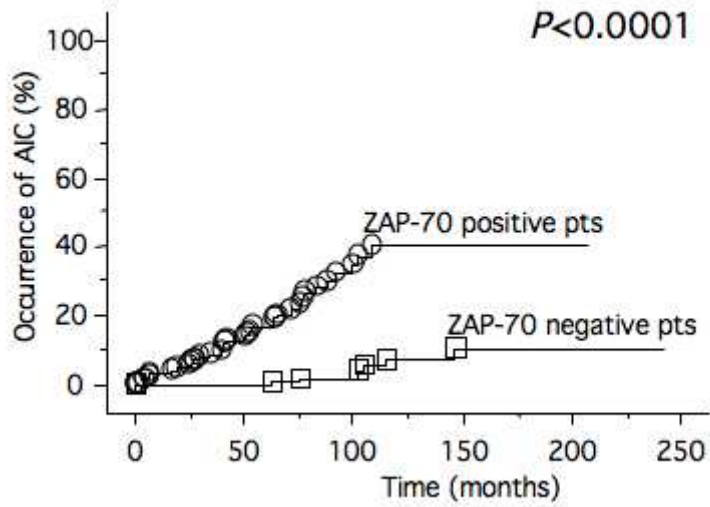
*Fisher's exact test.

#Mann-Witney.

§ chi-square test.

[°] Values available in 35 patients, ^{°°} values available in 25 patients

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

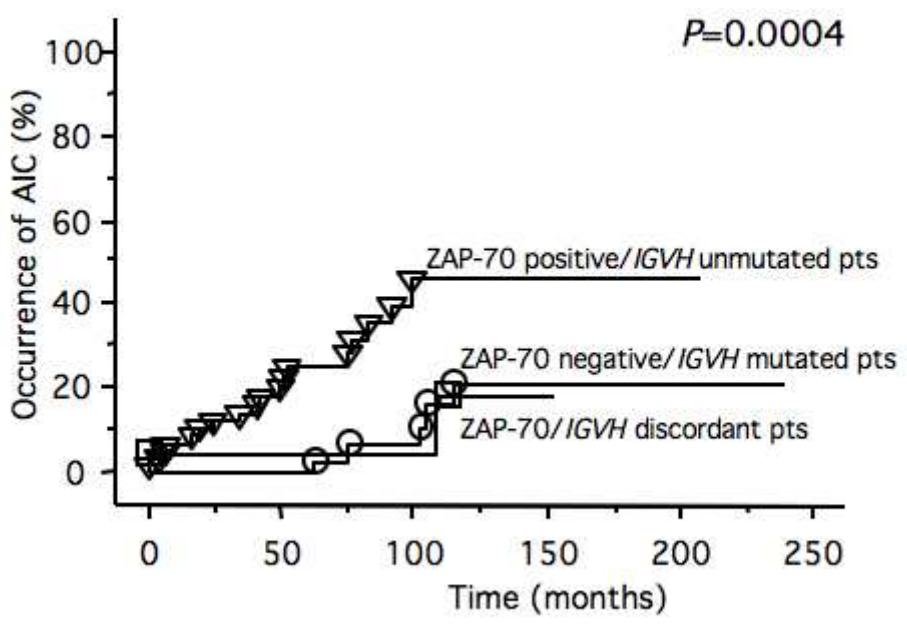


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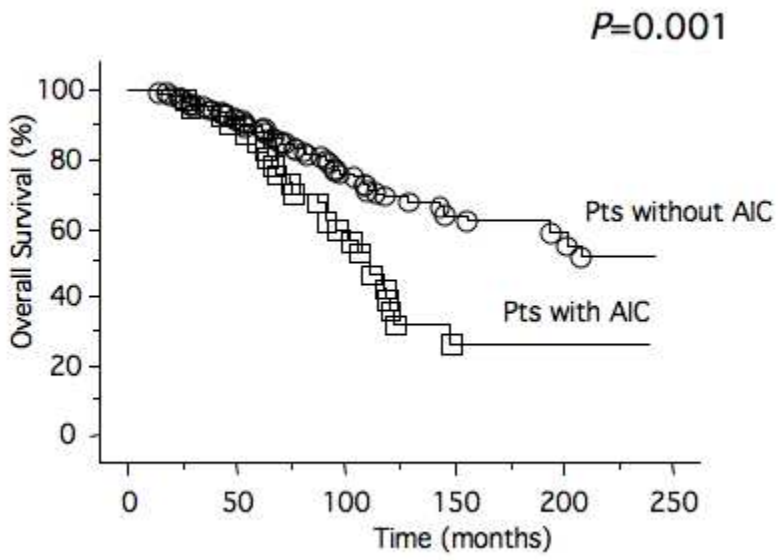
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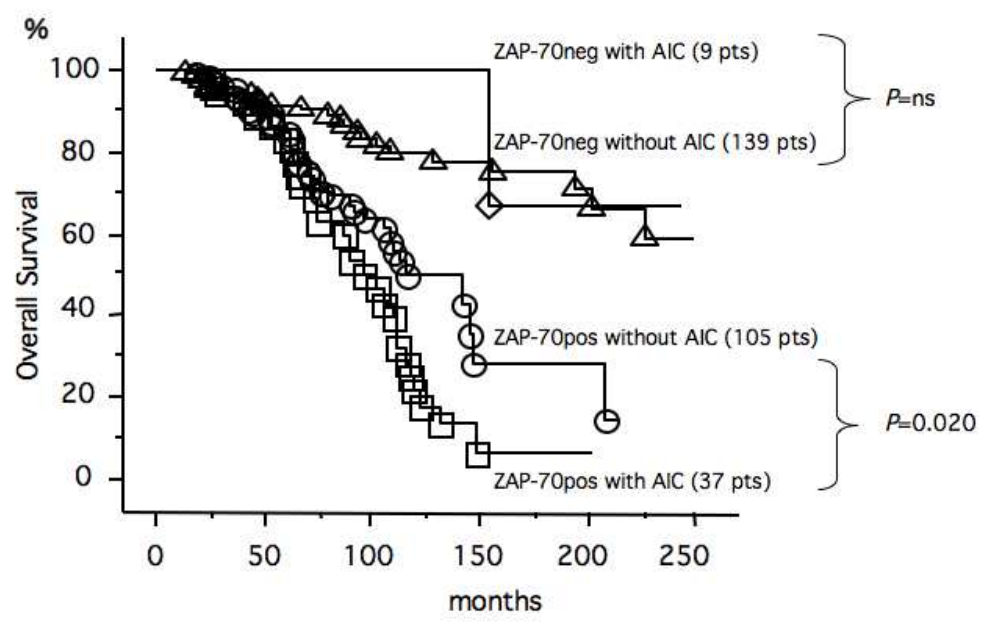
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