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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.

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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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In order to assess a possible correlation between ZAP-70 expression and other clinical and biological prognostic factors of CLL and the risk of AIC, 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 Haematology 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 of 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 males (64%) and 105 females, aged 33 to 85 years (median 63). At diagnosis 226 patients were classified as Binet stage A (78%), 51 stage B (18%) and 13 stage C (4%). The median follow-up from diagnosis was 80 months (range 12–240 months). The finding at diagnosis of anaemia or thrombocytopenia due to autoimmune phenomena was not considered as criteria for classifying patients as Binet stage C.

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 haemolysis (increased indirect bilirubin and/or LDH, decreased haptoglobin, increased absolute reticulocyte count), with no other cause for anaemia identified.

The diagnosis of AITP was made in patients with platelets <100 x10^9 with no evidence of hypersplenism, nor decreased BM platelet production (normal or increased megacariocytes on BM

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

The diagnosis of PRCA was based on Hb < 10 gr/dl, reticulocytopenia (< $10x10^9$/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, 33 with Fludarabine combined with Cyclophosphamide (FC) and 66 with 2 or more lines of therapy (mostly Chlorambucil followed by Fludarabine or FC).

ZAP-70 evaluation by IHC

In all patients 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*, 1984 (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).

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 ≥98% were considered unmutated, and those <98% mutated (22).

Statistical analysis

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The comparison of presenting clinical and laboratory features between the groups of patients with and without AIC was carried out with the χ^2 test, Fisher exact test or Mann-Whitney test as appropriate. OS was defined as the time from diagnosis of CLL to death or last follow-up. Time to AIC 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, in order to obtain unadjusted and adjusted Hazard Ratios (HR) and their 95% confidence intervals (95% CI). All tests were bilateral at $p<0.05$. The statistical analyses were performed using StatView (Abacus Concepts, Berkeley, CA).

RESULTS

Patient's data

Forty-six out of 290 patients (16%) developed AIC. Clinical and laboratory characteristics of these patients are listed in Table 1.

The most frequent form of AIC was AIHA due to 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 was recorded. The median age at occurrence of AIC was 67 years (range 41-85).

AIC occurred at diagnosis in 7/46 patients (15%) (4 AIHA, 2 AITP and 1 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%

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and 3%, respectively.

ZAP-70 evaluation by IHC on BM biopsies

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

ZAP-70 and CD38 evaluation by flow cytometry

ZAP-70 was expressed in ≥20% of B-CLL cells in 92/181 tested cases (51%). ZAP-70 expression evaluated both by IHC and flow cytometry was concordant in 160/181 cases (88%) and, particularly, in 21/22 patients with AIC evaluated with both techniques.

CD38 was expressed in ≥7% of B-CLL cells in 114/209 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 110/136 patients (81%). Of the 26 discordant cases, 16 (12%) were *IGHV* mutated and ZAP-70 positive, 10 (7%) unmutated 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 to those of the remaining patients are detailed in Table 1. 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 2**,** female sex, age >65 years, Binet stage B-C, PB lymphocyte count ≥ $30x10^9$/L, B2MG levels >2mg/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.

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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 due to the fact that was evaluated only in 136/290 patients and due to its high concordance rate with ZAP-70 expression in patients who developed AIC (92%).

Thirty-seven out of the 46 cases of AIC occurred in ZAP-70 positive patients (80%) and 9 in ZAP-70 negatives (20%). Characteristics of patients who developed AIC according to ZAP-70 expression were detailed in Table 3. In particular, ZAP-70 negative cases did not differ from ZAP-70 positives with respect to age and stage at diagnosis, sex, 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 unmutated, 7 (28%) mutated. Among the 136 patients tested for the mutational status the 7-years occurrence of AIC in the 59 ZAP-70positive/*IGHV*unmutated cases was higher (35%) as compared to that of the 51 ZAP-70negative/*IGHV*mutated patients (6%) and of the 26 patients discordant for the two prognostic parameters (4%) (P =0.0004) (Fig.2). The 2 discordant patients who developed AIC were both ZAP-70positive/*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 18/71 (25%) patients treated only with Chlorambucil, in 4/18 (22%) who received Fludarabine alone and in 6/79 (8%) treated with FC (χ^2 test: P=0.011).

Overall Survival

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We observed 87 disease or treatment related deaths (30%) and only 3 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 ≥ $30x10^9$/L (*P*=0.010), B2MG levels >2mg/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 Figure 3, patients who experienced AIC during the observation period had a 10-years 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

Haematological 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 haematological autoimmune complication in CLL (8). The prevalence of AIHA we observed (11%) was higher as compared to some recent studies (1-3,5); this finding could be related in part 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).

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In our series patients who developed AIC had lower male/female ratio, higher PB lymphocytes count, more frequent diffuse BM infiltration as compared to patients without AIC. Moreover they had more frequently unmutated *IGHV* genes and positive expression of ZAP-70. At multivariate analysis time to AIC was significantly correlated only with age >65 years and ZAP-70 expression. Other parameters reported as factors associated with AIC 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 due to 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 taken at diagnosis allowed the retrospective evaluation of patients with long follow-up. In our series ZAP-70 expression tested by IHC and flow cytometry respectively was concordant in 89% of cases. The possibility of discordance between the two methods probably did not influence our results: in fact in all but one of the 22 patients with AIC, analyzed with both tecniques, ZAP-70 expression results were concordant. The concordance rate of ZAP-70 expression evaluated by IHC and *IGHV* mutational status we observed (81%) was comparable with reported data, which range between 77% to 92%, depending on the different series and techniques (13-15,17,26).

We found ZAP-70 expression evaluated by IHC in 80% of patients with AIC; similar results are so far reported only in a small series of 5 AIHA (5). The 7-years cumulative incidence of AIC in ZAP-70 positive patients was 30% vs 4% of ZAP-70 negatives ($P<0.0001$).

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

The pathogenesis of AIC in CLL is still unclear. These autoimmune haematological phenomena are usually caused by polyclonal IgG autoantibodies produced by non-malignant B-lymphocytes and different from the Ig secreted by leukemic cells. In CLL, AIC are probably linked to the profound immune dysfunction that characterizes this disease, particularly in advanced phases. It has been hypothesized that autoimmune phenomena could be a consequence of a defect of regulatory T cells. Leukemic B cells might contribute to autoimmune complications through aberrant antigenic presentation (28). Since AIHA is reported to be more frequent in advanced CLL, an increased risk of AIHA (and generally of AIC) in patients with ZAP-70 expression could be related to a more aggressive disease behaviour and more profound immune defect. In CLL different types of AIC are probably strictly correlated. In fact in our series risk factors for both AIHA and AITP were similar and clinical manifestations not infrequently overlapping as in Evans Syndrome or in cases of AITP occurring after AIHA or viceversa.

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 appear 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 to 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

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patients with CLL (6). 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 appeared 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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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

parameters	*CLL without AIC (n=244)*	*CLL with AIC (n=46)*	*P*
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***
IGHV 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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Table 2. Effect of prognostic factors on time to AIC: univariate and multivariate analysis (Hazard Ratio - 95% CI)

Variable	*Unadjusted HR (95% CI)*	*Adjusted HR (95% CI)*
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 ≥ $30x10^9$/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 (≥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)**
IGHV unmutated (≥ 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

Parameters	*ZAP-70 neg* *9 pts*	*ZAP-70 pos* *37 pts*	*P*
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 (≥7% B cells)° (%)	2 (22)	18 (69)	**0.021***
IGHV unmutated (≥ 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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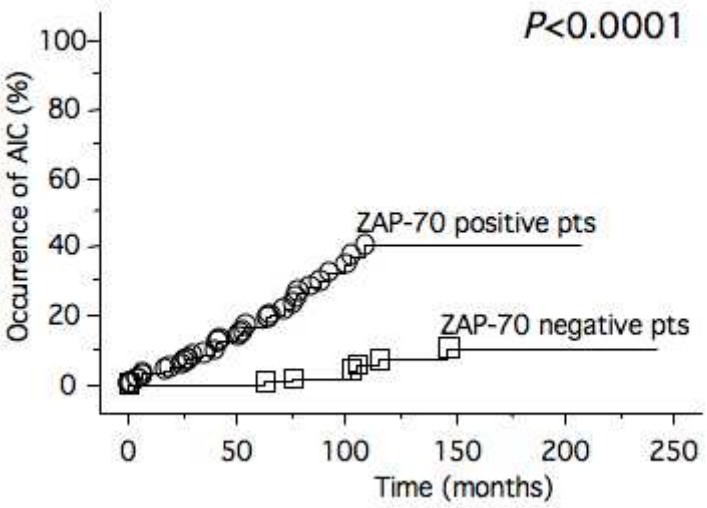

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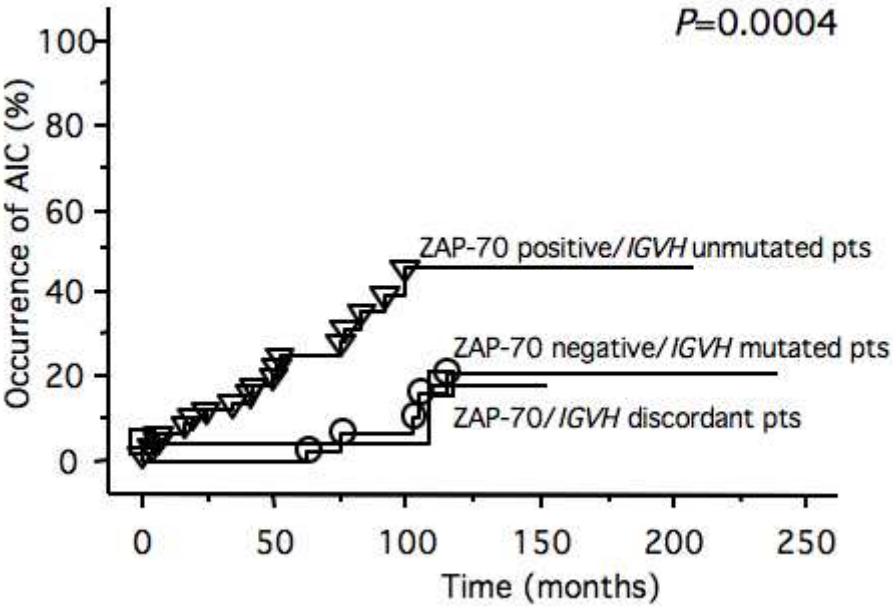

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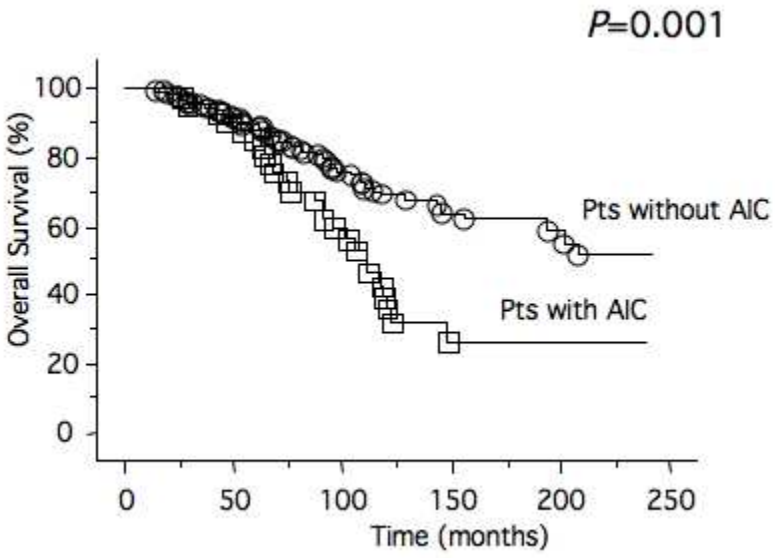

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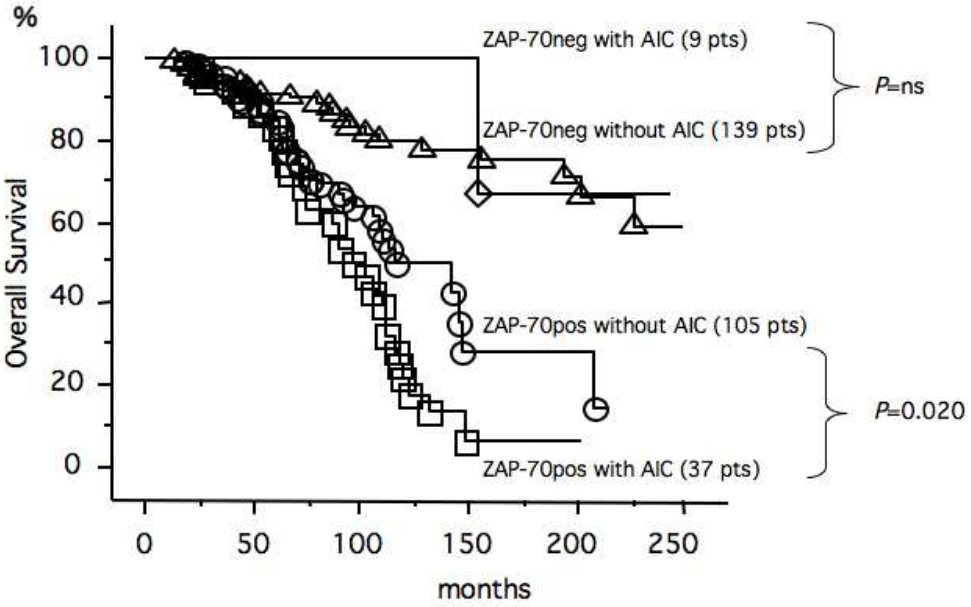

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