Fludarabine, Cyclophosphamide, and Mitoxantrone as Initial Therapy of Chronic Lymphocytic Leukemia: High Response Rate and Disease Eradication

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Abstract

Purpose: Fludarabine, cyclophosphamide, and mitoxantrone (FCM) results in a high response rate in previously treated patients with chronic lymphocytic leukemia (CLL). The aim of this study was to investigate FCM as frontline therapy in CLL.

Experimental Design: Sixty-nine patients under the age of 65 years with active CLL were treated. Patients received six cycles of fludarabine 25 mg/m^2 i.v. $\times$ 3 days, cyclophosphamide 200 mg/m^2 i.v. $\times$ 3 days, and mitoxantrone 6 mg/m^2 i.v. $\times$ 1 day. Treatment outcome was correlated with clinical and biological variables. The clinical significance of eradicating minimal residual disease (MRD) was also analyzed.

Results: The overall response, MRD-negative complete response (CR), MRD-positive CR, nodular partial response (PR), and PR rates were 90%, 26%, 38%, 14%, and 12%, respectively. Severe (grades 3 or 4) neutropenia developed in 10% of the patients. Major and minor infections were reported in 1% and 8% of cases, respectively. Median response duration was 37 months. Patients with del(17p) failed to attain CR. Patients achieving MRD-negative CR had a longer response duration and overall survival than patients with an inferior response. Low serum lactate dehydrogenase levels, low ZAP-70 expression, and mutated IgV_H genes predicted longer response duration. Finally, both low ZAP-70 and CD38 expression in leukemic cells correlated with MRD-negativity achievement.

Conclusion: FCM induces a high response rate, including MRD-negative CRs in untreated patients with active CLL. Treatment toxicity is acceptable. Both high ZAP-70 and increased CD38 expression predict failure to obtain MRD-negative response. Patients in whom MRD can be eradicated have longer response duration and overall survival than those with inferior response. These results indicate that FCM can be an ideal companion for chemoimmunotherapy of patients with CLL.

In spite of some progress in therapy, chronic lymphocytic leukemia (CLL) remains incurable (1). Over the last two decades, chlorambucil has been the mainstay for treatment of CLL. However, the complete response (CR) rate obtained with this agent is low (10%) and, besides symptom palliation, it is doubtful that chlorambucil has a significant effect on the natural history of the disease (2). Purine analogues, particularly fludarabine, are effective agents to treat CLL, resulting in a higher CR rate than chlorambucil or alkylating-based chemotherapies (20-40% versus 10%) and a longer disease-free interval; survival, however, is not prolonged (3–7). In addition, alemtuzumab results in higher response rates than chlorambucil in chemonaive patients (8).

Treatment of CLL is currently switching to purine analogue-based regimens with or without monoclonal antibodies (9–17). Although the benefits of these treatments in terms of overall survival have not yet been proven in randomized trials, these combinations induce higher response rates and a longer disease-free survival than alkylating agents or fludarabine alone (15, 18, 19). Notably, the newer treatment regimens

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induce a proportion of CR with undetectable minimal residual disease (MRD-negative CR), a situation that translates into a longer disease-free and overall survival (9, 12, 15, 20–22). Although immunochemotherapy is increasingly considered as the new standard for treatment of patients with CLL, the best combination chemotherapy to be given along with rituximab has not yet been identified.

Based on a number of *in vitro* and *in vivo* studies by others (23) and ourselves (16, 17), we developed a combination chemotherapy, including fludarabine, cyclophosphamide, and mitoxantrone (FCM) that has proved to be highly effective in patients with either relapsed or refractory CLL (9, 23).

Encouraged by these results, we conducted a prospective, multicentric phase II clinical trial to investigate FCM in patients under the age of 65 years with newly diagnosed CLL requiring therapy. In addition, the degree of response was characterized by assessing MRD. Finally, we analyzed the relationship between response and time to progression and different pretreatment clinical and biological variables, including genetic abnormalities, and ZAP-70 and CD38 expression. We report here the final results of this study.

Patients and Methods

Patients. Between July 2001 and August 2004, 69 patients with CLL were included in this study. Informed consent was obtained according to the ethical committees of the centers participating in this clinical trial and in agreement with the Spanish Ministry of Health requirements. The diagnosis of CLL was established according to the National Cancer Institute/CLL Working Group (NCI-WG) criteria (24).

Evaluation before treatment included clinical history, physical examination, WBC with differential count, liver and renal function tests, Coombs' test, and serum lactate dehydrogenase (LDH) and β_2-microglobulin levels. Bone marrow infiltration was assessed by needle aspiration and biopsy. Fluorescence *in situ* hybridization studies were done by using the commercial LSI p53/LSI ATM and LSI D13S319/LSI 13q34/CEP 12 Multicolor Probe Sets provided by Vysis (Downers Grove) using cutoff levels as previously described (25). ZAP-70 expression was analyzed in 39 patients by using the technique described by our group (26) and considered high when it was ≥20%. IgV_H mutational status was analyzed in 47 patients following previously described methods (26) and a sample was considered mutated if there was <98% homology to germ line IgV_H sequence. CD38 expression in CLL lymphocytes was considered increased when it was ≥30%.

Inclusion criteria were age below 65 years, active disease according to the NCI-WG criteria (24), and an adequate performance status (Eastern Cooperative Oncology Group 0-2). Patients with prior history of autoimmune phenomena or a positive Coombs' test, impaired renal or hepatic function, severe concomitant diseases, or pregnancy were excluded from the study. The median time from diagnosis to treatment was 20 months (range, 0.2-80 months), and the median follow-up for surviving patients was 30 months.

Therapy. FCM (fludarabine 25 mg/m^2 i.v. over 30 min for 3 days, cyclophosphamide 200 mg/m^2 i.v. over 1 h for 3 days, and mitoxantrone 6 mg/m^2 i.v. over 30 min on day 1) was given every 4 weeks as previously described (9). Patients received granulocyte colony-stimulating factor 300 μg daily for 7 days, and allopurinol 300 mg daily for 5 days starting on day 1. Treatment cycles were repeated every 4 weeks, depending on recovery of blood counts; if necessary, treatment was delayed until the neutrophil count was >1.5 × 10^9/L and the platelet count was more than 100 × 10^9/L. For patients with thrombocytopenia or anemia before starting FCM, treatment was only delayed if the values were <25% of the baseline counts. Doses of FCM were reduced if hematologic variables had not recovered to the levels previously described 6 weeks after the last cycle of therapy. Treatment was discontinued when absolute neutrophil count was <0.5 × 10^9/L or when the platelet count was <75% of the baseline value. Median number of FCM cycles administered was 6 (range, 3-6); 86% of patients received the entire planed therapy with no dose reductions. All patients received oral trimethoprim-sulfametoxazole twice weekly until 6 to 9 months after the end of the treatment as *Pneumocystis jirovecii* pneumonia prophylaxis.

Response criteria. Response was assessed 2 months after the end of the treatment using the NCI-WG criteria (24). Patients in CR with no detectable MRD were labeled as having MRD-negative CR. Bone marrow evaluation was not required in cases not attaining clinical CR. Reevaluation of the patients was done every 3 months. Whenever possible, bone marrow examination was done 6 and 12 months after treatment or when clinically indicated.

MRD analysis. MRD assessment was done using multiparametric flow cytometry and consensus PCR in peripheral blood and/or bone marrow at the time of response evaluation and every 3 months thereafter in patients who achieve a clinical CR or nodular partial response (nPR), until the MRD became detectable.

Whole peripheral blood samples were incubated with quadruple combinations of antibodies in a three-tube combination assay [CD20-FITC/CD79b-phycoerythrin/CD19-peridinin chlorophyll protein coupled to cyanine dye Cy5.5 (PerCP Cy5.5)/CD5-allophycocyanin (APC), CD22-FITC/CD23-phycoerythrin (PE)/CD19 PerCP Cy5.5/CD5 APC, and κ-FITC/λ-PE/CD19-PerCP Cy5.5/CD5-APC]. An additional tube was added to bone marrow samples to better discriminate CLL cells from mature and precursor B cells (CD20-FITC/CD38-PE/CD19 PerCP Cy5.5/CD5-APC). CLL cells were identified using the Paint-a-Gate software (Becton Dickinson) according to its characteristic phenotype (CD20dim/CD79bdim/CD19$^+$/CD5$^+$; CD22dim/CD23$^+$/CD19$^+$/CD5$^+$; CD20dim/CD38negative-dim/CD19$^+$/CD5$^+$; light chain restriction with dim expression identical to diagnosis in CD19$^+$CD5$^+$ cells). The value of MRD was the mean value of CLL cells observed in all the combinations excluding the κ/λ combination. The sensitivity (27, 28) of the test was 10^{-4}. Consensus PCR was done following protocols published elsewhere (29).

Statistical analysis. The CR rate in previously untreated patients receiving fludarabine as a single agent is around 20% (5). The estimated sample size to achieve a CR >40%, using a confidence level of 5% and a statistical power of 50%, was 68. The Fisher's exact test or χ^2 tests were used to analyze the association between patient characteristics and response. Survival time was measured from the time of initiation of therapy in all patients. Response duration was measured from the time of response evaluation to the progression of the disease. Time to treatment failure was considered from the time of initiation of the therapy until patients failed to respond to FCM and were removed from the study, suffered progressive disease, or died. Survival time, response duration, and time to treatment failure were analyzed by the method of Kaplan and Meier (30), and curves were compared by the log-rank test. All statistical tests were two sided and the significance level was 0.05.

Results

Patients' characteristic and response to therapy. Sixty-nine patients whose main characteristics are shown in Table 1 were included in the study. The overall response rate was 90% [95% confidence interval (95% CI), 83-97%]. The CR rate was 64% (95% CI, 52-75%): MRD-negative CR rate was 26% (n = 18; 95% CI, 16-38%), whereas the remaining cases (38%) were MRD-positive CRs. Ten patients (14%; 95% CI, 7-25%) achieved nPR. Eight patients (12%) were considered as in PR because of persistent lymphadenopathy (four cases) or a bone marrow infiltration >30% (three cases); one patient in clinical

CR and in whom a bone marrow biopsy could not be obtained was considered as in PR. Seven patients (10%) failed to respond; six of these patients did not show an adequate tumor reduction or presented persistent cytopenias, and the remaining patient developed Evan's syndrome 1 week after finishing treatment and was considered as a failure.

Pretreatment variables associated with CR achievement are listed in Table 1. Patients with enlarged spleen, advanced Rai's stage, increased serum LDH and β_2-microglobulin levels, diffuse bone marrow infiltration, and deletion 17p were less likely to achieve CR than those without these variables. Of note, none of the five patients having a 17p deletion obtained CR.

MRD was investigated by flow cytometry and consensus PCR in patients in clinical CR or nPR (n = 54). Among 44 patients in CR, 18 (41%) achieved MRD-negative status. Moreover, among 10 patients in nPR, one had no detectable MRD. Flow cytometry was more sensitive than consensus PCR in detecting MRD. Thus, half of the cases considered to be negative by consensus PCR resulted positive by flow cytometry. Patients achieving CR MRD-negative status had longer response and

Table 1. Response rate and response duration according to the main pretreatment characteristics of the patients

Variables	Distribution (%)	CR (%)	Patients in response (% at 3 y)
Gender			
Female	25	53	39
Male	75	68	53
Binet stage			
A	21	86	70
B	67	61	36
C	12	50	30
Rai stage			
0	3	100	100
I-II	74	71*	44
III-IV	23	40	51
B-symptoms			
No	67	71	51
Yes	33	50	43
Liver size			
Normal	82	65	46
Enlarged	18	67	52
Spleen size			
Normal	55	76*	49
Enlarged	45	50	33
Lymphocyte count			
$<50 \times 10^9$/L	42	75	49
$\geq 50 \times 10^9$/L	58	59	52
LDT (n = 45)			
>6 mo	60	74	62
≤6 mo	40	61	38
Serum LDH (n = 65)			
Normal	77	74*	66*
Increased	23	33	25
β_2-microglobulin (n = 60)			
<3 mg/L	49	79*	51
≥3 mg/L	51	53	43
BM pattern (n = 62)			
Nondiffuse	71	74*	49*
Diffuse	29	44	38
Genetic abnormalities (n = 50)			
del(13q)	34	56	49
+12	22	64	46
del(11q)	28	50	40
del(17p)	11	0*	—
Poor prognosis †	51	54	54
CD38 expression (n = 49)			
<30%	55	74	59
>30%	45	60	49
IgV_H mutational status (n = 47)			
Mutated	26	75	90*
Unmutated	74	57	40
ZAP-70 expression (n = 39)			
<20%	44	71*	82*
≥20%	56	59	33

Abbreviations: LDT, lymphocyte doubling time; BM, bone marrow pattern.
*$P < 0.05$.
† Cases with +12, del(11q), and/or del(17p).

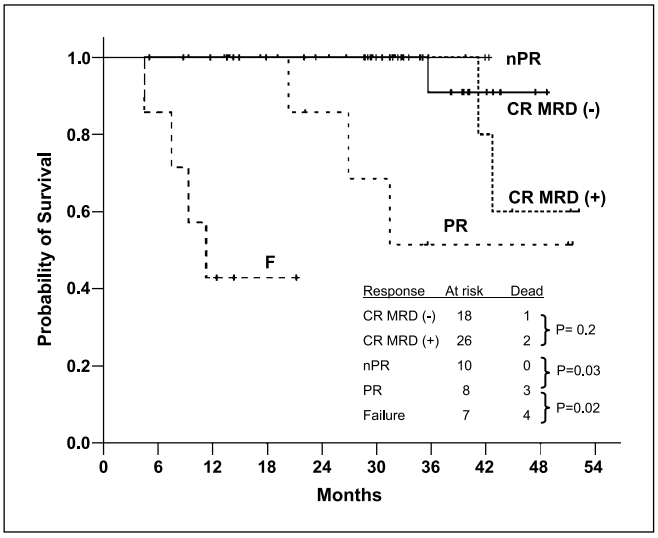

Fig. 1. Survival according to the degree of response. F, failure.

survival than those with an inferior response. Pretreatment variables associated with the achievement of MRD-negative CR status were ZAP-70 expression (low versus high, 13% versus 43%, respectively; $P = 0.033$) and CD38 expression (<30% versus ≥30%, 14% versus 44%, respectively; $P = 0.029$). Among responding patients, cytogenetic alterations did not correlate with the possibility of obtaining MRD-negative status.

Survival, time to treatment failure, and response duration. Overall survival was 70% (95% CI, 50-89%) at 4 years from the initiation of the treatment. At last follow-up, nine patients have died: seven patients because of progressive disease, one patient in PR developed an eventually fatal hepatitis B virus 2 months after the end of the treatment, and the remaining patient died due to a cerebrovascular hemorrhage while still in CR. There were no early deaths from infections. Median time to treatment failure was 44 months.

Survival time differed according to the degree of response. Survival probability at 3 years for patients achieving a MRD-negative CR, MRD-positive CR, or nPR was 100%; it was of 51% for patients achieving a PR, whereas patients failing to respond had a median survival of 11 months (Fig. 1).

Median response duration for all responding patients was 37 months. There were important differences in progression probability according to the degree of response. Whereas only one patient with MRD-negative CR (5%), six with MRD-positive CR (23%), and six with nPR (60%) progressed, all patients in PR progressed. At 2 years, probability of progression for patients with MRD-negative CR and MRD-positive CR was 9% and 20%, respectively. Moreover, probability of progression at 2 years for patients in nPR was 63%, whereas it was of 88% for patients in PR ($P = 0.03$; Fig. 2). Variables predicting response duration were serum LDH levels (median of 39 months versus 18 months for low and high LDH levels, respectively; $P = 0.038$), bone marrow infiltration pattern (nondiffuse, median of 36 months versus diffuse pattern, median 19 months; $P = 0.05$), IgV_H mutational status (mutated, median of 43 months versus unmutated, median 29 months), and ZAP-70 expression (<20%, median not reached versus >20%, median 24 months; $P = 0.0273$; Table 1; Fig. 3).

Probability of conversion from MRD-negative to MRD-positive status at 24 months was 41% (95% CI, 15-67%). Among all the variables analyzed, ZAP-70 expression in CLL cells ($P = 0.039$) and mutational status of IgV_H genes ($P = 0.04$), but not CD38 expression, correlated with the probability of MRD achievement (Fig. 4).

Toxicity. In general, FCM was well tolerated. Main toxicities are detailed in Table 2. Although some degree of neutropenia was observed in 31% of cycles, severe grade 3 to 4 neutropenia was only observed in 4% of the cycles. The WBC nadir, analyzed during the first cycle, was observed 7 days after the onset of treatment. Only one patient developed an autoimmune phenomenon (Evans' syndrome).

Infectious episodes, particularly fever of unknown origin, were recorded in 9% of the cycles, this frequency being equally distributed among neutropenic or nonneutropenic periods. Three patients presented hepatitis, two of them being fulminant B hepatitis that appeared at 3 and 4 months after the end of the chemotherapy, one of these cases being eventually fatal. These two patients had normal liver function before FCM treatment but one of them presented IgG antibodies against core B virus hepatitis. Finally, one patient presented an acute myeloid leukemia with *MLL* gene rearrangement 21 months after the end of treatment.

Dose reductions were necessary in 14% of the patients, mainly because of hematologic toxicity. Therapy was discontinued in three patients due to persistent neutropenia, diagnosis of bladder carcinoma, and development of Evans' syndrome, respectively.

Discussion

In patients with CLL, purine analogues given as single agents result in a higher response rate and a longer progression-free survival, but not a longer survival, than alkylators or anthracycline-based chemotherapy (3–7, 15).

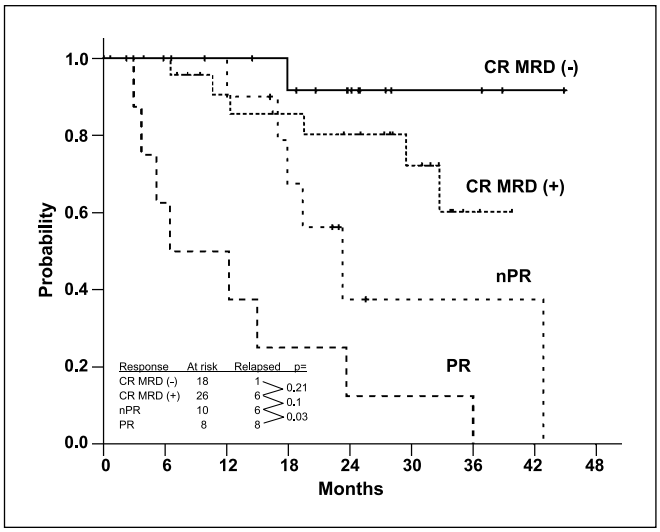

Fig. 2. Response duration according to the degree of response.

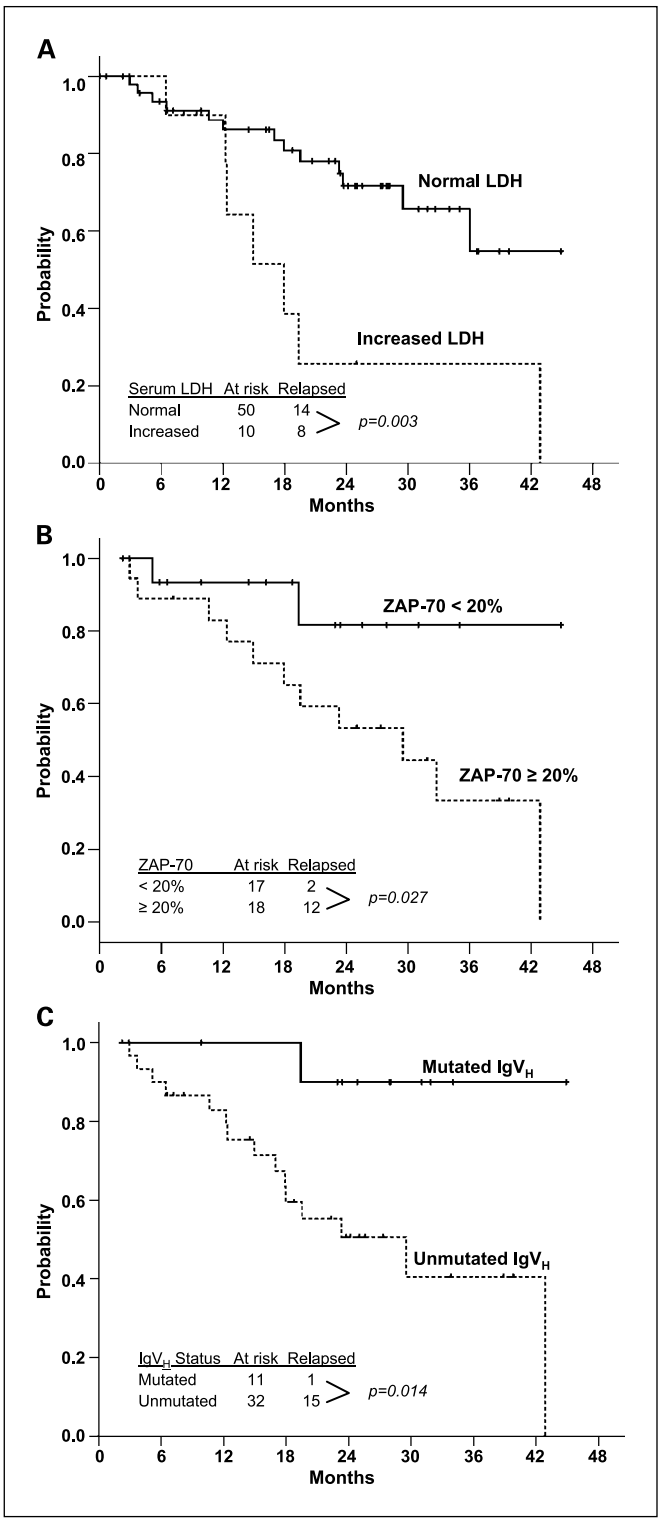

Fig. 3. Response duration according to serum LDH levels, ZAP-70 expression, and *IgV$_H$* mutational status assessed at the onset of FCM treatment.

To improve treatment results, different purine analogue–based combinations have been investigated; these regimens take advantage of the synergism between purine analogues, an effect particularly well shown for fludarabine, and other agents such as rituximab, cyclophosphamide, mitoxantrone, or oxaliplatin (9, 15, 18, 31–36). These treatments have proved to be more effective than purine analogues alone regarding response rate and response duration but there is no evidence yet of improvement in survival.

Immunochemotherapy (i.e., the combination of monoclonal antibodies with chemotherapy) is being increasingly considered

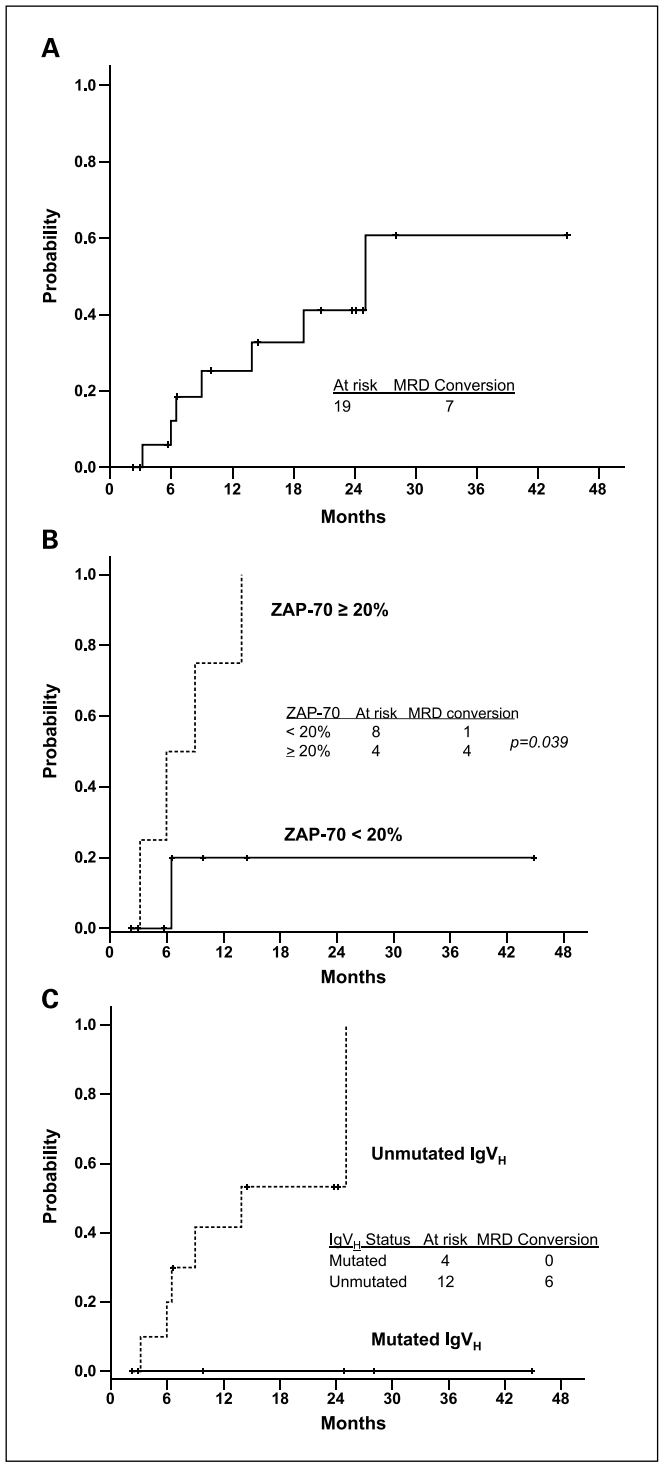

Fig. 4. Overall duration of the MRD-negativity status until MRD became positive. Patients with increased ZAP-70 expression or unmutated *IgV$_H$* had a shorter duration of the MRD-negative period.

Table 2. Hematologic and extrahematologic toxicity related to the FCM

Toxicity	Total	Grade 1-2	Grade 3-4
Hematologic*			
Neutropenia	31%	27%	4%
Thrombocytopenia	9%	9%	—
Anemia	13%	13%	—
Infections	9%	8%	1%
Infectious episodes by site			
Fever of unknown origin		12	
Upper respiratory tract		7	
Cutaneous abscess		5	
Pneumonia		2	
Urinary tract		1	
Diarrhea		1	
Sepsis (*Pseudomonas* spp.)		1	
Herpes zoster reactivation		1	
Nonhematologic †			
Nausea/vomiting	18%	16%	2%
Alopecia	5%	—	5%
Renal toxicity	1%	1%	—
Hepatic toxicity	6%	5%	<1% ‡
Others			
Associated neoplasia		Bladder carcinoma (during the 4th cycle) Laryngeal carcinoma (3 mo after end of therapy) Secondary *AML-MLL* gene rearrangement (21 mo after end of therapy)	

*Toxicity according to the NCI-WG.
† WHO classification expressed as percent of the cycles administered.
‡ Two patients developed fulminant B hepatitis at 2 and 3 mo after the end of the chemotherapy, respectively.

as the new standard to CLL treatment (21). Nevertheless, the superiority of a variety of immunochemotherapy regimens (e.g., rituximab + fludarabine; rituximab + fludarabine and cyclophosphamide; refs. 12, 21) over chemotherapy alone has not yet been shown in randomized clinical trials. In addition, and importantly, the best, most effective chemotherapy regimen to be combined with rituximab has not been identified either. In this regard, to investigate highly effective chemotherapy regimens for CLL is warranted.

The FCM combination was designed by our group on the basis of the synergistic effect of these drugs in inducing apoptosis in primary CLL cells *ex vivo* (16, 17). This regimen has proved to have an important antitumor activity in patients with heavily pretreated patients with CLL, including the achievement of MRD-negative CRs (9, 23).

The 64% CR rate obtained with FCM in the present study is one of the highest ever reported for any form of therapy in previously untreated patients with CLL. This CR rate is higher than those recently reported by the U.K. Leukemia Research Foundation (LRF CLL4), the German CLL Study Group, and the U.S. intergroup with a combination of fludarabine and cyclophosphamide (39%, 24%, and 23%, respectively) and only slightly inferior to the reported by the M.D. Anderson Group with the fludarabine, cyclophosphamide, and rituximab regimen (72%; refs. 12, 15, 18, 19). Our results also compare favorably with the 41% CR rate recently reported with pentostatin, cyclophosphamide, and rituximab (37). Comparing these clinical trials, however, it is difficult because patients included in the different studies are not necessarily equivalent. Interestingly, progression-free survival at 4 years was similar in all chemotherapy-only studies (50-55%; refs. 15, 18), whereas it was 77% for fludarabine, cyclophosphamide, and rituximab (12).

In our clinical trial, we assessed MRD by four-color flow cytometry, a technique that yields a high sensitivity in detecting MRD (38). The MRD-negative CR rate obtained with FCM compares favorably with that reported with other regimens, particularly when considering that in these later studies a less sensitive technique was used. As already shown in a number of studies, patients who achieve MRD-negative status after therapy have a much better outcome that those in whom MRD can be detected, including patients in CR by the classic NCI-WG criteria (9, 20, 22). This important concept is confirmed in our study in which patients achieving MRD-negative CR had longer response duration than patients with an inferior response.

A number of other findings in our study deserve comment, particularly regarding response predictors. Of note, patients with deletion 17q did not respond to FCM, this adding to the body of evidence on the ineffectiveness of fludarabine-based therapies in patients with this genetic lesion (18, 39, 40). However, no differences in the response rate were observed among patients with other abnormalities (i.e., normal, deletion 13q, trisomy 12, and deletion 11q).

Different studies have tried to link ZAP-70 and CD38 expression in leukemic cells with response to therapy and its duration, with contradictory results (41). We have found that both ZAP-70 and CD38 expression correlate with the probability of achieving MRD-negative status, which, if confirmed in other clinical trials and considering the increasing importance conferred to the eradication of MRD in patients' outcome, might be of critical importance in the design of risk-adapted therapies.

FCM was in general a well-tolerated regimen, with a low percentage of infections. Two patients presented with hepatitis. As a result of this and other similar observations, (42, 43) patients with a serologic proof of hepatitis B infection should

be excluded from fludarabine-based therapies. Finally, one patient presented an acute myeloid leukemia with *MLL*-gene rearrangement 21 months after the end of treatment, which is of interest when considering other reports expressing concern on the possible leukemogenic effect of fludarabine-based combinations (44).

In conclusion, in this clinical trial, we found that FCM is a well-tolerated regimen that produces a high response rate, including MRD-negative CRs, in previously untreated patients with active CLL. We also confirmed that eradicating MRD is an important treatment goal because MRD-negative responses convey longer response duration. Additional important observations were that ZAP-70 and CD38 expression correlated with the possibility of eradicating MRD. Finally, FCM warrants further investigation particularly in combination with monoclonal antibodies.

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