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Comparison between oral and intravenous fludarabine plus cyclophosphamide regime as front-line therapy in patients affected by chronic lymphocytic leukaemia: influence of biological parameters on the clinical outcome

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► To cite this version:

Luca Laurenti, Laura Padua, Michela Tarnani, Nicola Piccirillo, Paolo Falucci, et al.. Comparison between oral and intravenous fludarabine plus cyclophosphamide regime as front-line therapy in patients affected by chronic lymphocytic leukaemia: influence of biological parameters on the clinical outcome. *Annals of Hematology*, 2010, 90 (1), pp.59-65. 10.1007/s00277-010-1025-y . hal-00555319

HAL Id: hal-00555319

<https://hal.science/hal-00555319>

Submitted on 13 Jan 2011

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Editorial Manager(tm) for Annals of Hematology
Manuscript Draft

Manuscript Number: AOHE-D-10-00105R2

Title: Comparison between oral and intravenous fludarabine plus cyclophosphamide regime as front-line therapy in patients affected by chronic lymphocytic leukaemia: influence of biological parameters on the clinical outcome.

Article Type: Original Article

Keywords: Keywords: Chronic Lymphocytic Leukemia, Fludarabine, Cyclophosphamide, biological prognostic factors.

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Abstract: The fludarabine plus cyclophosphamide (FC) regimen was reported to be superior to chlorambucil or fludarabine alone in terms of complete response, overall response and progression free survival in previously untreated patients with chronic lymphocytic leukemia (CLL). In the present study, we compared the efficacy and toxicity of FC administered through oral and intravenous route in 65 untreated patients affected by advanced CLL. No statistical differences were noticed between the two routes of administration in terms of overall response (OR), progression free survival (PFS), time to retreatment (TTR) and overall survival (OS) of analyzed patients. We also assessed the influence on the clinical outcome of the mutation status of the immunoglobulin variable region heavy chain (IgVH) gene, of cytogenetic abnormalities and of the expression of ZAP70 and CD38 in patients primary samples. Among the 58 evaluable patients, 31 (53%) achieved a complete response and 18 (31%) a partial response. The median PFS was 35 months, median TTR was 42 months and median OS was not reached .after 45 months (range 1-161).

A significantly lower OR rate was noticed in patients with high-risk cytogenetic abnormalities (del 17p, del 11q). In this study, high-risk cytogenetic abnormalities and unmutated IgVH genes were independent predictors of TTR.

These results underline the importance of biological stratifications in front-line treatment of CLL patients. We confirm that FC is an effective regimen, with mild toxicities; it could be recommended for patients with low risk biological parameters, who represent in our experience about 30% of the total.

Response to Reviewers:

**Comparison between oral and intravenous fludarabine plus cyclophosphamide
regime as front-line therapy in patients affected by chronic lymphocytic
leukaemia: influence of biological parameters on the clinical outcome.**

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52 **Keywords:** Chronic Lymphocytic Leukemia, Fludarabine, Cyclophosphamide,
53 biological prognostic factors.
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Abstract

1 The fludarabine plus cyclophosphamide (FC) regimen was reported to be superior to chlorambucil or
2 fludarabine alone in terms of complete response, overall response and progression free survival in
3 previously untreated patients with chronic lymphocytic leukemia (CLL). In the present study, we
4 compared the efficacy and toxicity of FC administered through oral and intravenous route in 65
5 untreated patients affected by advanced CLL. No statistical differences were noticed between the two
6 routes of administration in terms of overall response (OR), progression free survival (PFS), time to
7 retreatment (TTR) and overall survival (OS) of analyzed patients. We also assessed the influence on
8 the clinical outcome of the mutation status of the immunoglobulin variable region heavy chain (IgVH)
9 gene, of cytogenetic abnormalities and of the expression of ZAP70 and CD38 in patients primary
10 samples. Among the 58 evaluable patients, 31 (53%) achieved a complete response and 18 (31%) a
11 partial response. The median PFS was 35 months, median TTR was 42 months and median OS was not
12 reached .after 45 months (range 1-161).
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23 A significantly lower OR rate was noticed in patients with high-risk cytogenetic abnormalities (del 17p,
24 del 11q).In this study, high-risk cytogenetic abnormalities and unmutated IgVH genes were
25 independent predictors of TTR.
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30 These results underline the importance of biological stratifications in front-line treatment of CLL
31 patients. We confirm that FC is an effective regimen, with mild toxicities; it could be recommended
32 for patients with low risk biological parameters, who represent in our experience about 30% of the
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Introduction

1 The purine analogue fludarabine inhibits the repair of DNA interstrand crosslinks caused by alkylating
2 agents [1,2]. The synergistic effect between fludarabine and cyclophosphamide prompted
3 investigation of a combined regimen of the two drugs for the treatment of patients affected by CLL.
4 Several studies, including two randomized trials, demonstrated that combined administration of
5 fludarabine and cyclophosphamide (FC) is superior to other standard treatments for CLL, leading to
6 the wide acceptance of this protocol as front-line therapy in patients with advanced disease [3-6].
7

8 More recently, a randomized controlled trial comparing FC versus FC plus Rituximab (FCR) showed
9 an improved overall response (OR) rate, progression-free survival (PFS) and overall survival (OS) in
10 patients receiving immunochemotherapy, and FCR can be considered the new standard for first-line
11 treatment of physically fit CLL patients [7]. Moreover, the same trial showed that the effect of FCR vs
12 FC in terms of OR rate is not improved in patients with mutated IgVH and standard risk FISH,
13 suggesting that biological risk stratification may be necessary to determine the optimal first-line
14 treatment in individual patients [8]. FCR also significantly improved PFS in previously treated CLL.
15 [9]
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17 We conducted a multicentre retrospective study to detect the efficacy and safety of FC as front-line
18 therapy of CLL, comparing two different schedules of treatment. In an effort to understand the impact
19 of the molecular prognostic features on response and outcome, we studied the influence of IgVH
20 mutation status, interphase cytogenetic abnormalities, and expression of ZAP-70 and CD38 on the
21 overall response rate, progression free survival, time to re-treatment (TTR), and overall survival.
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Materials and Methods

Patients and Treatment

This multicentre retrospective study included previously untreated patients with progressive CLL defined according to National Cancer Institute (NCI) criteria [10,11]. Patients with previous episodes of autoimmune haemolytic anaemia or positive direct/indirect Coombs test were excluded. The study was approved by the internal Ethical Committee at our Institution and all patients provided written informed consent.

Biological status of enrolled patients was tested at diagnosis or before starting treatment in all cases.

Thirty eight patients received oral fludarabine (30 mg/m²) and oral cyclophosphamide (250 mg/m²) and 27 patients received intravenous fludarabine (25 mg/m²) and intravenous cyclophosphamide (250 mg/m²); both treatments were administered for three consecutive days every 4 weeks for six cycles.

Patients receiving the oral formulation (os) or the intravenous administration (iv) were not hospitalized.

Patients receiving the oral or intravenous route in this study were randomly chosen. A slight reduction of the total dose of oral FC was adopted according to previous data from our institution [12].

Prophylaxis against herpes zoster and pneumocystis carinii was provided by administration of acyclovir 800 mg tid, and trimetoprim-sulfametoxazol 960 mg bid for 2 consecutive days every week, during the entire treatment period and 2 months after. Administration of G-CSF was used for grade IV neutropenia independently from the onset of fever. Treatment was delayed for up to two weeks in case of grade III-IV cytopenia, at day 28 of each cycle. Once the cytopenia was reduced to grade I or II, treatment was reinstated with a 25% dose reduction of both drugs for all subsequent cycles. A further 25% dose reduction was performed in case of grade III-IV cytopenia in subsequent cycles. Treatment was discontinued in case of progressive disease, autoimmune haemolytic anemia, autoimmune thrombocytopenia or pure red cell aplasia (PRCA) .

Study End Points

The efficiency of the treatment was assessed according to the NCI-WG response criteria [10, 11]. Progression-free survival, time to re-treatment and overall survival were defined from the date when therapy was started to the date of progression, re-treatment and death or last follow-up, respectively.

1 The primary end-point of the study was the response rate and safety of oral and intravenous treatment
2 with fludarabine and cyclophosphamide. The secondary end-point was an evaluation of the influence
3 of biological parameters on OR rate, PFS, TTR and OS.

4 *Fluorescent In Situ Hybridization*

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6 Interphase FISH studies were performed according to the manufacturer's instructions on peripheral
7 blood samples using a set of four commercial probes (Abbott, Wiesbaden-Delkenheim, Germany) [13].
8 An alpha satellite DNA probe CEP12 directly labelled with SpectrumOrange was used to detect
9 aneuploidy of chromosome 12. LSI p53 together with CEP17 alpha satellite DNA probes labelled
10 with SpectrumOrange and SpectrumGreen, respectively, were used to evaluate chromosome deletion
11 at 17p13.1. LSI RB and LSI ATM, both labelled with SpectrumOrange, were used to detect
12 chromosome deletion at 13q34 and 11q22-23, respectively. Prior to hybridization, the slides were
13 dehydrated with three ethanol washes (70%, 85%, and 100%) and air-dried. A 10 microL probe
14 mixture was added to each slide, and the slides were covered with a coverslip. The slides were then
15 denatured using a Vysis Hybrite set (Vysis Inc.) at a melting temperature of 75°C for 5 minutes and
16 then hybridized at 37°C overnight. The coverslips were removed, and the slides were washed for 2
17 minutes in 0.4 x SSC at 73°C and then in 2 x SSC at room temperature for 2 minutes. Nuclei were
18 counterstained with 46-diamindino-2-phenylindole dihydrochloride and cover again with a coverslip.
19 A minimum of two hundred consecutive, qualifying, interphase nuclei were analysed using a Zeiss
20 AxiosKop 2 plus fluorescent microscope. The normal cut-off value was established by analysing 10
21 peripheral blood samples from normal blood subjects.

22 *IgVH gene mutational analysis*

23 Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll gradient centrifugation
24 (Amersham Biosciences, Uppsala, Sweden). Total cellular RNA was isolated from PBMCs using the
25 Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed
26 using random hexamers and then amplified with the polymerase chain reaction (PCR) using a
27 degenerate VH framework 1 (FWR1) primer in combination with C μ , C δ and C γ reverse primers.
28 Samples that failed to amplify with these combinations were amplified with a mixture of forward
29 primers complementary to leader sequences of VH family 1 to 6. PCR products were purified with the
30 QIAquick PCR purification kit (Qiagen, Valencia, CA) and either sequenced directly or cloned with
31

1 the PCR Cloning Plus kit (Qiagen) [14]. Sequencing analysis was performed with the BigDye
2 Terminator v3.1 Cycle Sequencing kit on an ABI 3100 genetic analyzer (Applied Biosystems, Foster
3 City, CA). Candidate germ-line genes were assigned by searching the VBASE directory. Sequences
4 with less than 2% differences from germ-line VH sequences were considered unmutated.
5

6 *Flow Cytometry*

7
8 Fifty microliters of whole blood in EDTA containing approximately 5×10^5 cells were incubated at
9 room temperature in the dark for 20 minutes with CD3 PE-Cy5, CD56 PE-Cy5, CD5 FITC and CD19
10 PE-Cy7 to identify surface antigens. After incubation, samples were centrifuged at 1500 rpm for 5
11 minutes. Cell pellets were resuspended in 100 microL of fixation medium A (caltag Laboratories,
12 Burlingame, CA, USA) and incubated at room temperature in the dark for 15 minutes. Then cells were
13 pelleted again at 1500 rpm for 5 minutes, resuspended in 100 microL of permeabilization medium B
14 (Caltag Laboratories) and incubated with the ZAP-70 monoclonal antibody at room temperature in the
15 dark for one hour. The ZAP-70 antibody was R-phycoerythrin conjugated (clone 1E 7.2, Caltag
16 Laboratories). Samples were washed twice by centrifugation at 1500 rpm for 5 minutes and
17 resuspended in 500 microL of phosphate-buffered saline. Finally, 1×10^4 fixed cells were analyzed by
18 flow cytometry (BD FACSCanto, Becton Dickinson) using the gating strategy described by Crespo et
19 al [15]. Cases expressing more than 20% ZAP-70 positive CLL B-cells were defined as positive.
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22 CD38 analysis was performed in the setting of routine cellular immunophenotyping using PE-
23 conjugated anti-CD38 from Becton Dickinson (San Jose, CA). Peripheral blood mononuclear cells,
24 separated from heparinised venous blood by density gradient centrifugation (Histopaque-1077, Sigma
25 Diagnostics, St Louis, MO) were analyzed for surface expression of CD19/CD5/CD38 and
26 CD19/CD5/CD23 by triple colour immunofluorescence. Isotype-matched negative controls were used
27 in all the assays to determine positive from negative results. Flow cytometric analyses were performed
28 on a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with
29 argon and red diode lasers. The CellQuest software system (Becton Dickinson Immunocytometry
30 Systems) was used to acquire and to analyze data. Cases expressing more than 30% CD38 positive
31 CLL B-cells were defined as positive [16].
32

Statistical analysis

1 First we analyzed the impact of the two different routes of administration (oral / iv) on the following
2 outcomes: Overall Response rate defined as achievement of complete response (CR), partial response
3 (PR), and nodular PR; PFS defined as the time interval from the first day of treatment until disease
4 progression; TTR calculated from the first day of treatment until initiation of a new treatment; overall
5 survival time, calculated from the first day of treatment until death from any cause. For non-
6 responders and progressive disease, date of progression was when no response or progressive disease
7 was recorded.
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10 The following parameters were used: sex, age (< 65 years vs >= 65 years), Binet stage (B/C),
11 cytogenetic abnormalities (high risk, HR vs standard risk SR cytogenetics.), IgVH mutational status,
12 expression of ZAP-70 and CD38. We chose to consider patients with 11q deletion and 17p deletion
13 together as a high-risk cytogenetic group because of the small number of patients with these
14 abnormalities (8 patients with del 17p and 7 patients with del 11q). Response to treatment was also
15 included for PFS/TTR/OS outcomes.
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18 The chi-square test was used to analyze the categorical variables. Univariate survival analysis was
19 performed using the Kaplan-Meier method and Logrank test. The Cox proportional hazards model and
20 the logistic regression model were used to test the association of the covariates with outcomes of
21 PFS/TTR/OS and OR rate, respectively. Only variables that in univariate analysis reached a *P value
22 <0.25 were included in the model. The models were built using the Backward Stepwise (Wald)
23 procedure. The results are expressed as Hazard Ratios (HRs) and 95% confidence intervals (CI95%).
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25 Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS), release
26 16.0 for Windows. A *P value less than 0.05 was considered significant.
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Results

Patient characteristics

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2 Sixty-five previously not treated patients with progressive CLL from three different Italian institutions
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4 were enrolled in the study. Thirty-eight patients received oral schedule treatment at the Catholic
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6 University Medical Centre; 27 patients were enrolled from a medical center that had adopted iv
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8 schedule of FC combination: 15 patients were treated at the Viterbo Hospital and 12 patients at the
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10 San Giovanni Rotondo Hospital. Median time between the diagnosis and the beginning of therapy was
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12 27 months (range 1-137). Forty-eight patients were male and 17 were female, with a median age of 65
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14 years (range, 44 to 78 years). Fifty-two patients were in stage B/II, 4 in stage C/III, and 9 in stage
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16 C/IV. Thirty-three patients had not mutated and 18 had mutated IgVH genes; in 14 patients the
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18 mutation status was not determined. Fifteen patients (23%) had the 'high risk' cytogenetic
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20 abnormality del(11q22.3) or del(17p13.1). Twenty eight patients expressed ZAP-70 (cut-off 20%) and
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22 23 patients were CD38-positive (cut-off 30%). (Table 1).
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Response

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28 Fifty eight patients were evaluable for response to treatment. Seven patients did not complete the
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30 planned treatment, two of them because of onset of immune thrombocytopenia that developed after 2
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32 and 4 cycles; one because of PRCA that developed after the first cycle and one because of skin
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34 toxicity that developed after 2 cycles. The other three patients died during treatment, two from
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36 pneumonia that occurred in the setting of grade IV neutropenia after the first and second cycle,
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38 respectively, and one from sudden cardiac death after 3 cycles of therapy.
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41 Thirty one patients (53%) obtained a complete response and 18 (31%) a partial response, with an OR
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43 rate of 84%. Four patients showed stable disease and five patients experienced disease progression
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45 after 2, 3, 4 (two patients) and 6 cycles respectively. No statistical differences were observed when
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47 comparing the OR in the group of patients treated with iv FC (83% OR) and those treated with oral
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49 FC (85% OR).
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51 The high-risk cytogenetic patients group included 8 patients with del 17p and 7 with del 11q. We
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53 noticed 4 response to treatment in each cytogenetic alteration, even if 3 of four 17p deletion patients
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55 showed less than 20% positive cells.
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57 Univariate analysis showed cytogenetic abnormalities as the only factor influencing the overall
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59 response rate (*P=0,016). CD38 expression and stage of disease were also included in the multivariate
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model because of a *P value <0.25 in the univariate analysis, but only high risk cytogenetic abnormalities were identified as an independent prognostic parameter (HR 0,093; CI 0,015-0,580). The IgVH, ZAP-70 and CD38 categories did not show statistical differences in terms of OR rate (table 2).

PFS, TTR and OS

Median PFS was 35 months (range 2-118). In univariate analysis,, response to treatment was significantly associated with PFS (*P< 0.001). IgVH mutation status (*P=0.132) and FISH (*P=0.058) was also included in the multivariate model because of a *P value <0.25, but only response to therapy was identified as an independent prognostic parameter (table2).

Median TTR was 42 months (range 2-118). Response to treatment (*P<0.001), IgVH mutation status (*P=0.026), cytogenetic abnormalities (*P=0.007) and age (*P=0.25) were investigated in the multivariate model; response to treatment, IgVH and cytogenetic abnormalities emerged as independent prognostic factors (table 2, figure 1 and 2).

Median OS was not reached after 45 months (range 1-161). During the study period, 19 patients died. In univariate analysis only response to treatment achieved a statistically significant value (*P<0.001), whereas response to treatment and age emerged as independent prognostic factors from the multivariate analysis (table 2).

Hematological and extra-hematological toxicity

Twelve patients developed grade III and 21 patients developed grade IV neutropenia (37% after iv FC and 29% after oral FC). Two of these patients, both of them from the iv FC arm, died because of pneumonia after the first and the second cycle of therapy. Three patients developed grade III or grade IV anemia that required red blood cell transfusions. Two patients developed immune thrombocytopenia after the 2nd and 4th cycle and one patient developed pure red cell aplasia after the 1st cycle, which regressed after treatment with steroids and Rituximab.

As for extra-hematological toxicity, nausea was reported by 18 patients and 8 of them also experienced vomiting; as expected these complications were predominantly observed in the oral FC group. One patient developed fever of unknown origin after the first cycle of chemotherapy, which spontaneously resolved without hospitalization. One patient showed severe skin toxicity due to chemotherapy and discontinued treatment after 2 cycles. One patient died after 3 cycles of therapy

from sudden cardiac death due to congenital abnormalities of the coronary arteries discovered during autopsy (table 3).

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A large multicenter study recently demonstrated that fludarabine plus cyclophosphamide is superior to chlorambucil or fludarabine alone as front-line treatment of CLL with respect to complete response, overall response and progression free survival [4]. In addition, FC was found to be superior to chlorambucil or fludarabine alone in all age groups, including patients older than 70 years, suggesting that FC can be considered the standard treatment for chronic lymphocytic leukaemia. The results of this study confirmed our preliminary data and other published studies [3-6,12,17] regarding the efficacy and safety of fludarabine plus cyclophosphamide as front line therapy in patients affected by chronic lymphocytic leukemia (table 4).

Table 4 shows a similar response to therapy among five data series on the FC regimen, even if the dose, timing and route of administration were different in the reported studies. The table also underlines differences in PFS between different studies that could be explained by lower cumulative dose of chemotherapy or different schedule of administration. No comparison of TTR and OS is possible because of unavailability of comparable data.

In our study we did not detected any significant difference in the clinical outcome between the iv FC and the oral FC groups, even though the total dose of fludarabine and cyclophosphamide was higher in the iv FC group. Patients who received oral combination were previously reported in our published series. In that series, we adopted a total dose of FC slightly decreased compared to other published studies in order to reduce the side effects of the drugs and to avoid hospitalization in elderly patients with comorbidities. The higher intravenously dose could explain why patients from the iv FC group experienced more often grade III/IV neutropenia and more severe infections, including two fatal pneumonias. On the other side, nausea and vomiting occurred more often among patients receiving oral FC.

Results from a large non randomized trial of FCR and preliminary results of a randomized, controlled trial comparing FC versus FCR showed an improved response rate and progression free survival in patients who received the immunochemotherapy regimen, so FCR has become the new standard first-line treatment for physically fit CLL patients [7, 18]. The same study reported that the effect of FCR vs FC on the OR rate is not higher in patients with IgVH mutated CLL and standard risk FISH [8]. In the monoclonal antibody era, when the association with chemotherapy shows certain benefits on clinical outcome but also more haematological and extra haematological toxicities, a stratification of the biological risk of patients prior to treatment should be considered. In our series, we found that the

1 'high risk' cytogenetic abnormalities are independent predictors of a lower OR rate as well as a
2 shorter TTR. In addition, unmutated IgVH genes are significantly associated with a shorter TTR,
3 whereas expression of CD38 and ZAP-70 has no significant impact on clinical outcome.

4 As expected, response to treatment was an independent predictor of longer PFS, TTR and OS, while
5 age was predictive of longer OS in our study. Biological risk stratifications demonstrated that patients
6 with "high risk" cytogenetic abnormalities and germ line IgVH genes have a shorter time to
7 retreatment following FC chemotherapy, suggesting that high risk untreated younger patients affected
8 by progressive CLL should be candidates to receive chemo-immunotherapy. Conversely, a consistent
9 group of low risk CLL patients emerged from the biological stratification. These patients display
10 mutated IgVH and standard risk FISH and they respond to FC with an high OR rate and a prolonged
11 TTR. A good compliance to treatment and the strong tolerability exhibited by this substantial
12 proportion of patients argue that they could benefit from FC alone. FCR can be also employed to treat
13 these patients, but haematological and extra-hematological complications can arise in elderly patients
14 (> 70 aa) as elsewhere reported.
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Figure 1

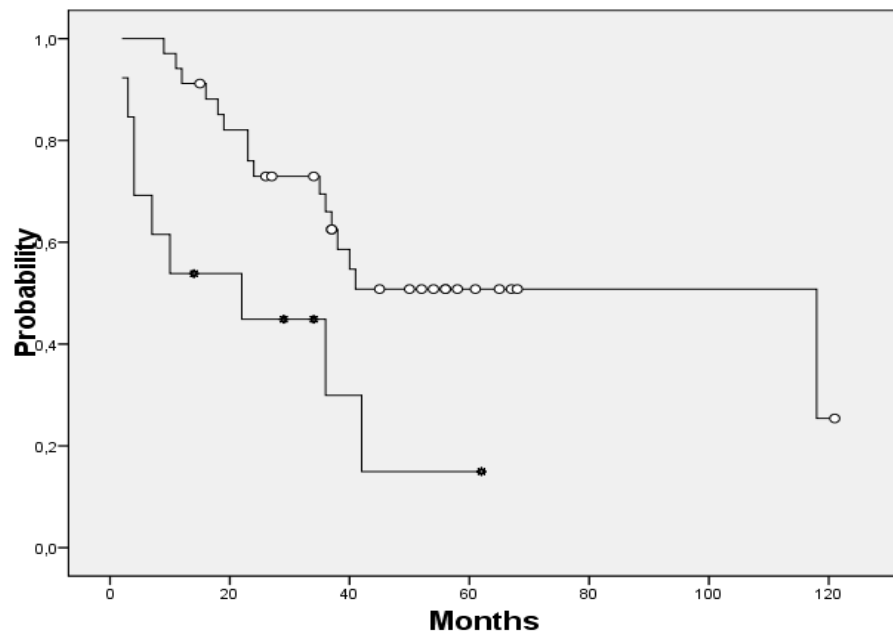


Figure 2

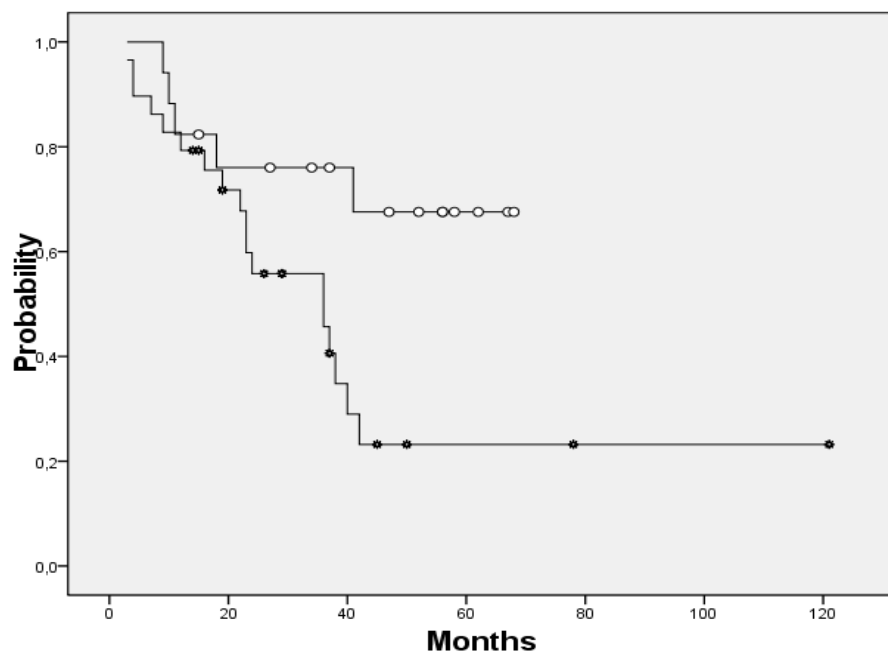


Figure Legend

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Table 2 Multivariate analysis

	<i>HR</i>	<i>OR</i> <i>CI95%</i>	<i>P*</i>	<i>HR</i>	<i>TTP</i> <i>CI95%</i>	<i>P*</i>	<i>HR</i>	<i>TTT</i> <i>CI95%</i>	<i>P*</i>	<i>HR</i>	<i>OS</i> <i>CI95%</i>	<i>P*</i>
Age: >65 vs <65 years		Not included			Not included			Not included		3,656	1,046-12,780	0,042*
Overall R vs Nr		Not applicable		0,102	0,035-0,293	<0,001*	0,080	0,026-0,248	<0,001*	0,071	0,021-0,239	<0,001*
FISH: High vs standard risk	0,093	0,015/0,580	0,011*	2,250	0,929-5,440	0,072*	2,587	1,024-6,540	0,045*		Not included	
IgVH: NM vs M		Not included		2,195	0,860-5,599	0,1*	3,512	1,164-10,598	0,026*		Not included	
CD38: pos vs neg	7,767	0,694-86,896	0,096*		Not included			Not included			Not included	
ZAP-70: pos vs neg		Not included			Not included			Not included			Not included	

Legend: R=response; Nr=not response

Table 1 Clinical and prognostic characteristics of CLL patients

Features	Number of patients	OS FC (38 patients)	IV FC (27 patients)
Males	48	26	22
Females	17	12	5
Stage BII	52	33	19
Stage CIII/IV	4/9	1/4	3/5
FISH HR	15	7	8
FISH SR	36	27	9
IgVH NM	33	18	15
IgVH M	18	14	4
CD38 \geq 30%	23	11	12
CD38 $<$ 30%	37	27	10
ZAP70 \geq 20%	28	18	10
ZAP70 $<$ 20%	28	19	9

Table 3 Treatment related toxicity according to NCI

Kind of toxicity	Number of patients	
	OS FC (38 patients)	IV FC (27 patients)
Hematological (grade III/IV)		
Anemia	2/1	0
Neutropenia *	7/11	5/10
Immune thrombocytopenia	2	0
PRCA	0	1
Extra-hematological		
Nausea	14	4
Vomiting	7	1
Pneumoniae* (in grade IV neutropenia) death	0	2 (exitus)
Sudden cardiac death	1 (exitus)	0
Skin toxicity	1	0
Febrile unknown neutropenia	1	0

*: p=ns

Table 4: Comparison of our experience and published data of FC front-line treatment

	Laurenti (65 patients)*	Cazin [17] (75 patients)	Catovsky [4] (196 patients)	Flinn [6] 83 patients	Eichhorst [3] 180 patients
Treatment schedule	os FC/iv FC	os FC	os FC/iv FC	iv FC	iv FC
Mg/m² for cycle of fludarabine	90/75	150	120/75	100	90
Mg/m² for cycle of ciclophosphamide	750/750	1000	750/750	600	750
OR rate (CR/PR)	84 (53/31)	80 (53/27)	94 (38/57)	74 (23/51)	94 (16/78)
Median (months) PFS/TTR/OS	35/42/nr at 45 months	60/nr/nr at 7 y	43/nd./nr at 5 y	31,6/nd/nd	48/nd/nd

Legend: nr=not reached nd=not done

* unpublished data

Editor in chief

We re-submit to your attention the article entitled 'Comparison between oral and intravenous fludarabine plus cyclophosphamide regime as front-line therapy in patients affected by chronic lymphocytic leukaemia: influence of biological parameters on the clinical outcome'.

According to the Reviewer 2 :

- 1) The study is retrospective as reported in Methods section.
- 2) We added the explanation of the route choice in Methods section
- 3) The use of FCR regimen front line or in relapsed patients was more stressed in the Introduction section.

Detailed comments:

- 1 Reference n.7 was replaced as suggested
- 2 The results from REACH study were mentioned in Introduction section, even if a comparison between FC versus FCR in previously treated patients was not discussed because it was not the topic of the paper
- 3,4,5 We modified table 4 as requested (references, we removed reference 12 and specified 'unpublished' for our data)

Reviewer 3

- 1) We clarify in Method section and in discussion the choice of lower dose of oral FC.
- 2) We added references for the methods used for FISH, IgVH and CD38.
- 3) Informed consent was just specified in Methods section
- 4) We added in the last part of the discussion the value of FCR schedule even in low risk patients. To note that our patients are more elder than CLL8 patients and also at the ASH meeting 2009 Hallek showed more toxicity in terms of neutropenia (III-IV) and infections in elderly (> 70 aa) patients.

Reviewer 4

- 1) As suggested we added the explanation of the route choice in Methods section and we also reported the differences between these two groups of patients in table 1.
- 2) We added in Method section (statistical analysis) why del 11q and del 17p were both considered as high risk cytogenetics. Also we reported in Results the same percent of response in each subgroup (del 11q and 17p), even if 3 of four patients with del 17p showed a percentage of positive cells less than 20%.
- 3-4) Biological state of enrolled patients was tested at diagnosis or before starting of treatment in all patients depending on the time of diagnosis. No patients were studied after treatment. We added in Method sections.
- 5) We did not find statistical differences in the quantities of infectious ($p=0,088$) and neutropenia ($p=0,49$) between peroral and intravenous administration; we also added in table 3 statistical results.
- 6) We reported in Results section the median time between diagnosis and therapy; moreover as reported in point 3-4) we stressed that biological study was done at diagnosis or before the treatment. We think that acquired cytogenetic alterations (IgVH and ZAP70 were immutable during the time), according to the literature, don't develop without prior therapy; anyway no patients were studied after starting treatment.

Minor points were changed as requested

We hope it will be re-considered for publication in your journal.

Thanking you in advance for your attention.

Sincerely yours,

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