ARTICLE

Impaired nodal shrinkage and apoptosis define the independent adverse outcome of *NOTCH1* mutated patients under ibrutinib therapy in chronic lymphocytic leukemia

Giovanni Del Poeta,[1] Annalisa Biagi,[1] Luca Laurenti,[2] Annalisa Chiarenza,[3] Federico Pozzo,[4] Idanna Innocenti,[2] Massimiliano Postorino,[1] Francesca Maria Rossi,[4] Maria Ilaria Del Principe,[1] Riccardo Bomben,[4] Paolo de Fabritiis,[1] Antonio Bruno,[1] Maria Cantonetti,[1] Francesco Di Raimondo,[3] Antonella Zucchetto[4] and Valter Gattei[4]

[1]Division of Hematology, Department of Biomedicine and Prevention, University Tor Vergata, Roma; [2]Division of Hematology, Università Cattolica del Sacro Cuore, Roma; [3]Division of Hematology, Ferrarotto Hospital, Catania and [4]Clinical and Experimental Hematology Unit, Centro di Riferimento Oncologico di Aviano (CRO), IRCCS, Aviano (PN), Italy.

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ABSTRACT

The introduction of agents inhibiting the B-cell receptor-associated kinases such as ibrutinib has dramatically changed treatments algorithms of chronic lymphocytic leukemia (CLL) as well as the role of different adverse prognosticators. We evaluated the efficacy of ibrutinib as a single agent, in a real-life context, in 180 patients with CLL mostly pretreated, recruited from three independent cohorts from Italy. Patients received 420 mg oral ibrutinib once daily until progression or occurrence of unacceptable side effects. Seventy-three patients discontinued ibrutinib for progression or for adverse events. *NOTCH1* mutations (*NOTCH1 M*) were correlated with a reduced redistribution lymphocytosis, calculated at 3 months on ibrutinib (P=0.022). Moreover, *NOTCH1 M* patients showed inferior nodal response at 6 months on ibrutinib compared to *NOTCH1* wild-type patients (P<0.0001). Significant shorter progression free survival (PFS) and overall survival (OS) were observed in *NOTCH1 M* patients (P=0.00002 and P=0.001). Interestingly, *NOTCH1 M* plus a lower BAX/BCL-2 ratio identified a CLL subset showing the worst PFS and OS (P=0.0002 and P=0.005). In multivariate analysis of PFS and OS, *NOTCH1 M* were confirmed an independent prognosticator (P=0.00006 and P=0.0039). In conclusion, *NOTCH1 M* are strongly associated with a lower BAX/BCL-2 ratio, consistent with defective apoptosis, lower redistribution lymphocytosis and lower nodal shrinkage under ibrutinib treatment, this last paramter being responsible for partial responses, subsequent relapses, as well as shorter PFS and OS. Either new small molecule combination approaches or antibodies targeting *NOTCH1* could be future therapeutic options for *NOTCH1 M* patients.

Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent adult leukemia in Western countries and it is characterized by an extremely heterogeneous clinical course. New molecular aberrations with negative prognostic value in CLL, such as *NOTCH1*, *MYD88*, *TP53* and *SF3B1* gene mutations, were identified in the last decade mainly thanks to the advent of next-generation sequencing (NGS).[1,2] In particular *NOTCH1* mutations (*M*) are found in 10-14% of patients at diagnosis with frequency increasing with disease progression and during transformation to Richter syndrome.[3] Furthermore *NOTCH1 M* are associated with the presence of trisomy 12 and with high CD49d expression which are negative prognostic factors in CLL. *NOTCH1 M* are also associated with an increased activation of the NF-kB pathway, promoting tumour cell proliferation and survival.[4] *NOTCH1 M* were shown to affect

Correspondence:

GIOVANNI DEL POETA
g.delpoeta@tin.it

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the response to chemo-immunotherapy in CLL. In the CLL8 study Stilgenbauer *et al.*[5] demonstrated that patients carrying *NOTCH1 M* did not benefit of the addition of rituximab to standard fludarabine and cyclophosphamide chemotherapy. Moreover, it has emerged that *NOTCH1 M* are associated with decreased duration of response in a large series of relapsed/refractory (R/R) patients treated with venetoclax.[6]

In a recent study, Tissino *et al.*[7] have demonstrated that patients with CLL whose cells were characterized by high CD49d expression, underwent reduced lymphocytosis and inferior nodal response after treatment with ibrutinib. Several reports confirmed that in CLL the balance between the pro- and anti-apoptotic members of the BCL-2 family determines chemotherapy sensitivity and cell survival.[8,9] Noteworthy, we demonstrated that a low BAX/BCL-2 ratio had an additive negative prognostic impact in both *TP53 M* and *NOTCH1 M* patients with CLL treated with chemo-immunotherapy.[10] The recent introduction of novel B-cell receptor inhibitors such as ibrutinib and idelalisib and of novel potent oral BH3 peptidomimetics such as venetoclax in clinical practice, prompted us to evaluate the clinical impact of both *NOTCH1 M* and BAX/BCL-2 ratio in patients treated with targeted oral therapies and in particular in those treated with ibrutinib.

The aims of this study were: i) to analyse the correlations between *NOTCH1 M* and other biological parameters including CD49d expression and the BAX/BCL-2 ratio; ii) to address the impact of *NOTCH1 M* both on redistribution lymphocytosis and on nodal responses after treatment with ibrutinib; iii) to evaluate the impact of *NOTCH1 M* and BAX/BCL-2 ratio on the overall response rate (ORR) to ibrutinib, progression free survival (PFS) and overall survival (OS); iiii) to assess whether *NOTCH1 M* could be considered an independent prognostic factor.

Methods

Study design and patients

In this study we retrospectively analysed 180 patients with CLL exposed to treatment with ibrutinib. Patients were recruited from three independent cohorts from Italy (Rome Tor Vergata University, Rome Cattolica Sacro Cuore University and Catania Ferrarotto Hospital), between 2014 and 2019. Informed consent was obtained in accordance with the Declaration of Helsinki. The study was performed under the Institutional Review Board of the Centro di Riferimento Oncologico (IRCSS) of Aviano (approval numbers: IRB-05-2010 and IRB-05-2015).

Patients were 122 males and 58 females with a median age of 69 years (range, 36-90). According the modified Rai staging system,[11] 134 patients had an intermediate risk and 46 a high risk stage. All these parameters were considered at the time ibrutinib was initiated.

All patients received 420 mg oral ibrutinib (Imbruvica; Janssen, Beerse, Belgium) once daily until progression or occurrence of unacceptable side effects. Median number of previous chemotherapy regimens were two (range, 0-4). Patients receiving first-line ibrutinib were 26 (14.4%), of whom 24 of 26 (92%) were *TP53* mutated. Median follow-up was 25 months (range, 10-61). Seventy-three patients (40.6%) discontinued ibrutinib either for progression (n=42) or for adverse events (n=31) (Table 1): 32 patients were subsequently treated with venetoclax (11 for toxicity [grade 3 or 4 World Health Organization] and 21 for progression of disease), five patients were treated with idelalisib, and the remaining 36 patients received other lines of chemotherapy (n= 12) or no therapy (n=24). The clinical characteristics of patients are reported in Table 1. The clinical assessment of patients with CLL to establish diagnosis and response to therapy were based both on the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) criteria.[12] The clinical impact of *NOTCH1 M* and BAX/BCL-2 ratio on ibrutinib treatment was evaluated by measuring the kinetics of absolute lymphocyte counts (ALC), the reduction of lymphadenopathy, and the clinical outcome, as defined by ORR, PFS and OS.

Chronic lymphocytic leukemia characterization

Flow cytometry was employed for immunophenotypical CLL characterization and was performed with FACSCalibur or FACSCanto I flow cytometer. BCL-2 and BAX oncoproteins were analysed by flow cytometry in samples taken before starting ibrutinib. BAX/BCL-2 ratio was calculated dividing mean fluorescence intensity (MFI) of BAX by MFI of BCL-2 on CLL cells, as previously described.[10] The threshold of positivity was set at ≥1.5. Immunoglobulin heavy-chain variable region gene (*IGHV*) mutational status was performed by NGS, as previously described.[13,14] *TP53* exons 2 to 11 mutational status and *NOTCH1* exon 34 and 3' untranslated (UTR) region mutational status were analysed by NGS, as previously described.[4,7] CLL samples were considered

Table 1. Patient characteristics (n = 180)

	No. of patients/Total cases (%)
Observation time	2014-2019
Median age, y (range)	69 (36-90)
Males	122/180 (68)
Modified Rai stage	
Intermediate	134 (74)
High	46 (26)
Number of previous regimens	
0	26/180 (14.5)
1	56/180 (31.1)
2	74/180 (41.1)
3	22/180 (12.2)
4	2/180 (1.1)
NOTCH1 mutation	65/180 (36.1)
BAX/BCL-2 ratio <1.50	74/113 (65.5)
Trisomy 12	23/179 (13)
11q deletion	35/179 (20)
TP53 mutations/17p deletion	66/178 (37.1)
UM *IGHV*	123/175 (70.3)
CD38 ≥ 30%	54/113 (47.8)
CD49d ≥ 30%	108/179 (60.3)
Median follow up (months)	25 (10-61)
Response to ibrutinib therapy	
Complete response	33/180 (18.3)
Partial response	51/180 (28.3)
Partial response with lymphocytosis	81/180 (45.1)
Stable disease/No response	15/180 (8.3)
Discontinuation	73/180 (40.6)
Progression	42/180 (35)
Toxicity	31/180 (65)
Richter Syndrome	13/180 (7.2)
Progression-free Survival at 2 years	80%
Overall Survival at 2 years	84%
Overall Survival at 4 years	71%

y: years; IGHV: immunoglobulin heavy-chain variable region gene. No.: number.

mutated for *NOTCH1 i.e., NOTCH1 M*, if exceeding a variant allele frequency (VAF) of 1%.[15,16]

Redistribution lymphocytosis and nodal response

The redistribution lymphocytosis was calculated as percent variation of ALC over the baseline values. Nodal response was calculated as percent reduction in sum of the product of diameter (SPD) values on the major lymph node regions over the baseline measurement, as reported previously.[17]

Additional details on the employed procedures and methods are reported in the *Online Supplementary Materials and Methods*.

Results

NOTCH1 mutations and BAX/BCL-2: correlations with other biological parameters

Sixty-five patients were *NOTCH1 M* (65 of 180, 36.11%), with VAF levels >1 (*Online Supplementary Table 2S*). With regard to the distribution of VAF levels, 21 patients had VAF between 1% and 10%, seven patients between 10.5% and 20% and 37 patients above 20%. Fifty-six *NOTCH1 M* cases bore a single mutation, eight cases two mutations and one case three mutations. *NOTCH1 M* cases were classified as follows: 45 delCT, six frameshift other than delCT (FS), 8 3'-UTR and six considering both missense (one) and nonsense (five) mutations (*Online Supplementary Table S2*). Seventy-four patients showed a BAX/BCL-2 ratio lower than 1.5 (74 of 113, 65.5%). *NOTCH1 M* were significantly associated with SPD ratio<1.5: in fact, 34 of 38 *NOTCH1 M* patients showed BAX/BCL-2 ratio less than 1.5 (P=0.0001). Moreover, *NOTCH1 M* were strongly correlated with CD49d expression: 51 patients were both *NOTCH1 M* and CD49d ≥30% (P=0.0001). Furthermore, a significant correlation was found between a lower BAX/BCL-2 ratio and CD38>30% (41 of 54; P=0.030) as well as between CD38>30% and *NOTCH1 M* (27 of 38 patients; P=0.0004) (Table 2; *Online Supplementary Table S3*).

Trisomy 12 was confirmed to be strongly correlated with *NOTCH1 M* (18 of 23; P=0.0002). There was only a trend towards significant association between *NOTCH1 M* and *IGHV UM* status (48 of 62; P=0.08). On the other hand, *IGHV UM* status was correlated with lower BAX/BCL-2 ratio (58 of 81; P=0.030). *TP53 M* and/or del17p were found in 66 of 178 patients (37.1%). Noteworthy, 23 of 178 patients (13%) were simultaneously *NOTCH1* and *TP53* mutated. The distribution of clinical and biological prognostic factors according to *NOTCH1 M* is shown in Table 2. The distribution of prognostic factors according to the BAX/BCL-2 ratio and CD38 was obtained in 113 patients from Rome and shown in Table 2 and the *Online Supplementary Table S3*.

Relevance of *NOTCH1* mutations as biological prognostic parameter

The mean peripheral lymphocyte percentage change from baseline, calculated at 3 months on ibrutinib, was lower in *NOTCH1 M* patients than in *NOTCH1* wild-type (*WT*) patients (14% *vs*. 54%; P=0.022, Mann-Whitney test), thus confirming a reduced redistribution lymphocytosis (Figure 1A).

Moreover, the mean percent SPD change, calculated at 6 months on ibrutinib, was lower in *NOTCH1 M* patients than in *NOTCH1 WT* patients (53% *vs*. 80%; P<0.0001, Mann-Whitney test), confirming a significant poor nodal response (Figure 1B). Moreover, we compared *NOTCH1 M* plus lower BAX/BCL-2 ratio *versus NOTCH1 M* plus higher BAX/BCL-2 ratio with respect to redistribution lymphocy-

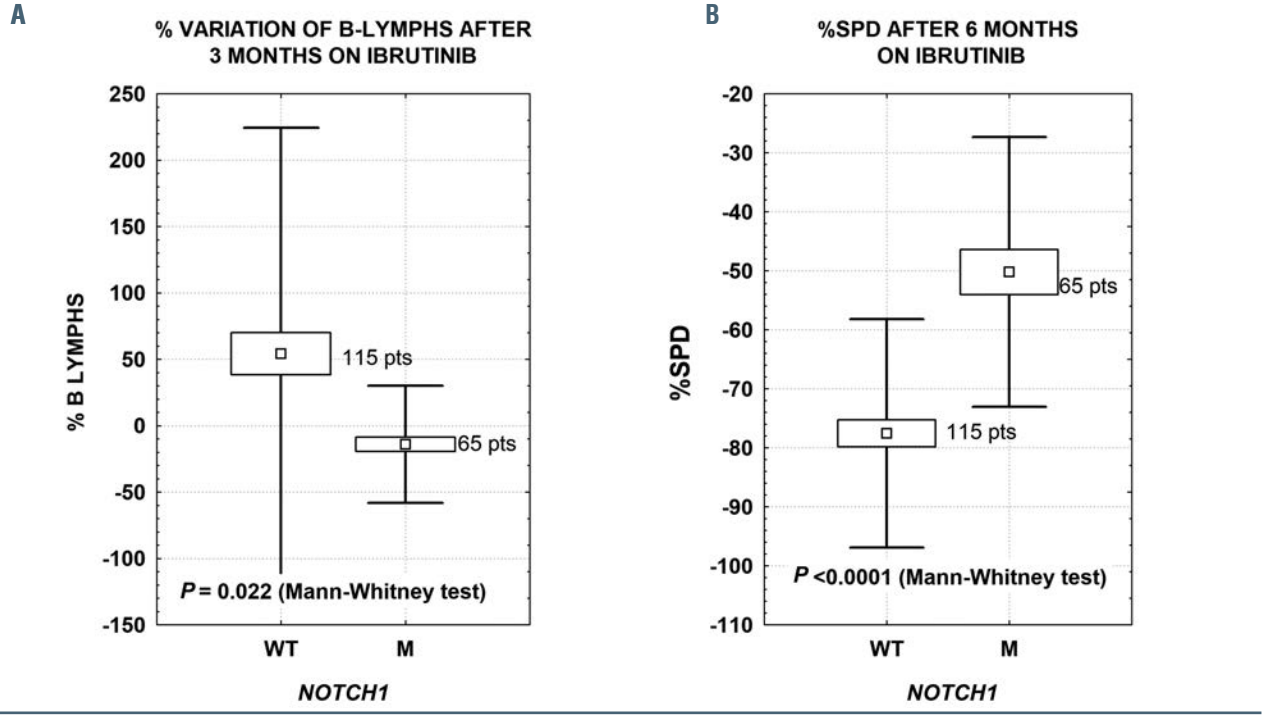

Figure 1. Box plots by *NOTCH1* wild-type and mutated groups showed significant lower redistribution lymphocytosis after 3 months on ibrutinib treatment in *NOTCH1 M* patients (A) and equally lower sum of the product of diameter (SPD) values after 6 months in *NOTCH1 M* patients (B). pts: points; WT: wild-type; M: mutated; B LYMPHS: B lymphocytes.

Table 2. Distribution of prognostic factors in chronic lymphocytic leukemia according to *NOTCH1* mutations

Parameter	*NOTCH1* Mutated	*NOTCH1* Wild-type	*P*¶	n§	4-year OS,%	*P**	4-year PFS, %	*P**
Age								
<60 years	31	54	0.52	180	85	0.29	81	0.72
>60 years	34	61			95		89	
Sex								
Male	45	77	0.44	180	122	0.71	115	0.55
Female	20	38			58		55	
Mod-Rai								
Intermediate	10	30	0.32	180	140	0.81	40	0.33
High	55	85			40		130	
Lines of therapy								
≤ 2	55	101	0.54	180	156	0.004	148	0.002
> 2	10	14			24		22	
CD49d								
<30%	14	57	0.0001	179	71	0.23	68	0.045
>30%	51	57			108		101	
CD38								
<30%	11	48	0.0004	113	59	0.52	56	0.36
>30%	27	27			54		50	
FISH								
Normal/del13q	24	46	0.0002	179	71	0.49	70	0.46
+12, 11q-,17p- del11q, del17p)	41	68			107		98	
IGHV								
Mutated	14	38	0.080	175	52	0.76	48	0.036
Unmutated	48	75			123		117	
TP53								
Mutated	23	43	0.52	178	66	0.028	59	0.022
Wild-type	40	72			112		109	
BAX/BCL-2 ratio								
<1.5	34	40	0.0001	113	74	0.013	67	0.0019
>1.5	4	35			39		39	

¶ Fisher exact tests were performed to evaluate the association between *NOTCH1* mutations or wild-type and other prognostic factors. § Values refer to the number of cases analysed for a given feature. **P*-values were calculated by the log-rank test in univariate analysis. PFS: progression-free survival; OS: overall survival; FISH: fluorescence *in situ* hybridisation; IGHV: immunoglobulin heavy-chain variable region gene.

tosis and lymph node shrinkage. No significant differences were found between these two subsets (*Online Supplementary Figure S3*).

NOTCH1 mutations, BAX/BCL-2 ratio and their impact on clinical outcome

According to clinical endpoints, ORR was 91% [complete response (CR): 18%, partial response (PR): 28%, PR with lymphocytosis (PR-L): 45%] (Table 1). The estimated 2-year and 4-year OS were 84% and 71%, respectively (Table 1; *Online Supplementary Figure S4*). Noteworthy, OS was longer in patients previously treated with one line of chemo-immunotherapy before ibrutinib (*P*=0.02, *Online Supplementary Figure S5*). PR and PR-L were significantly correlated with *NOTCH1 M* (30 of 65 and 22 of 65, respectively; *P*=0.00001, *Online Supplementary Table S4*). Of note, PR, PR-L and chemoresistance were also associated with lower BAX7BCL-2 ratio (23 of 29, 33 of 52 and nine of nine, respectively; *P*=0.002, *Online Supplementary Table S5*). Interestingly, discontinuation due to disease progression was more frequent in *NOTCH1 M* patients than in *NOTCH1 WT* patients (*P*=0.034, *Online Supplementary Table S4*). Significant shorter PFS and OS were observed in *NOTCH1 M* patients (34% *vs*. 76% and 56% *vs*. 83% at 3 years, respectively; *P*=0.00002 and *P*=0.001; Figure 2A and B). There were no significant differences among VAF range 1-10%, 10.5-20% and above 20% with respect to PFS and OS, as shown in the *Online Supplementary Figures S6 and S7*. Moreover, we restricted the analysis of *NOTCH1* to the relapse setting only (154 of 180 patients) obtaining similar significant results regarding PFS and OS (*Online Supplementary Figures S8 and S9*).

Table 3. Multivariate Cox regression analysis

Parameter	PFS 168 patients HR	PFS 168 patients *P*	OS 178 patients HR	OS 178 patients *P*
NOTCH1 M	3.89	0.00006	2.64	0.0039
>2 lines of therapy	2.88	0.0040	2.43	0.015
TP53 M	2.05	0.028	1.94	0.047

PFS: progression-free survival, OS: overall survival: M: mutant; HR: hazard ratio.

Additive prognostic properties of NOTCH1 mutations and BAX/BCL-2 ratio

In order to obtain a better refinement in the prognostic assessment of PFS and OS, we combined the values of the BAX/BCL-2 ratio with those of *NOTCH1*. Within the subset of 113 patients from Rome, shorter PFS and OS were detected both in patients with *NOTCH1 M* (46% *vs*. 83% and 68% *vs*. 86% at 3 years, respectively; *P*=0.0019 and

P=0.031, *Online Supplementary Figure S10A and B*) and with lower BAX/BCL-2 ratio (60% *vs*. 97% and 72% *vs*. 94% at 3 years, respectively; *P*=0.019 and *P*=0.013, Figure 3A and B). Therefore, higher or lower BAX/BCL-2 ratio combined with *NOTCH1 WT* or *NOTCH1 M* identified two subsets of patients, the former with the best prognosis and the latter with the worst prognosis with respect to both PFS (97% *vs.* 42%; *P*=0.0002, Figure 4A) and OS (94% *vs.* 63%;*P*=0.005, Figure 4B), confirming the true additive prognostic properties of these two prognosticators.

Multivariate analysis

The clinical impact of *NOTCH1* as independent prognosticator was checked by multivariate Cox proportional hazards analysis applied to models including two other prognosticators proven to be significant in univariate analysis (Table 2). With respect to PFS, *NOTCH1 M* (*P*=0.0002) were confirmed as an adverse independent prognostic factor (*P*=0.00006) together with >2 previous lines of therapy (*P*=0.004) and *TP53 M* (*P*=0.028) (Table 3). Similarly, in a multivariate analysis of OS, *NOTCH1 M* retained an independent prognostic value (*P*=0.0039) together with >2 previous lines of therapy (p=0.015) and *TP53 M* (p=0.047) (Table 3). *NOTCH1 M* and >2 previous lines of therapy were confirmed as independent prognosticators for PFS (*P*=0.035 and *P*=0.015, respectively) also in a model that included the BAX7BCL-2 ratio, available in a smaller subset of cases (n=113, *Online Supplementary Table S6*). Conversely, in the same subset of patients, no factor emerged as independent prognosticator for OS (*Online Supplementary Table S6*).

Discussion

In the present study we evaluated the efficacy of ibrutinib treatment in the high-risk *NOTCH1 M* CLL group and correlated *NOTCH1 M* to BAX/BCL-2 ratio, a value reflecting the susceptibility of cells to apoptosis. Efficacy of ibrutinib remained high at 4-year follow-up in almost all pre-treated patients with CLL, with 71% of patients alive and progression free, similarly to other studies.[17] Moreover, ibrutinib was more effective in patients previously treated with only one line therapy, compared to patients previously treated

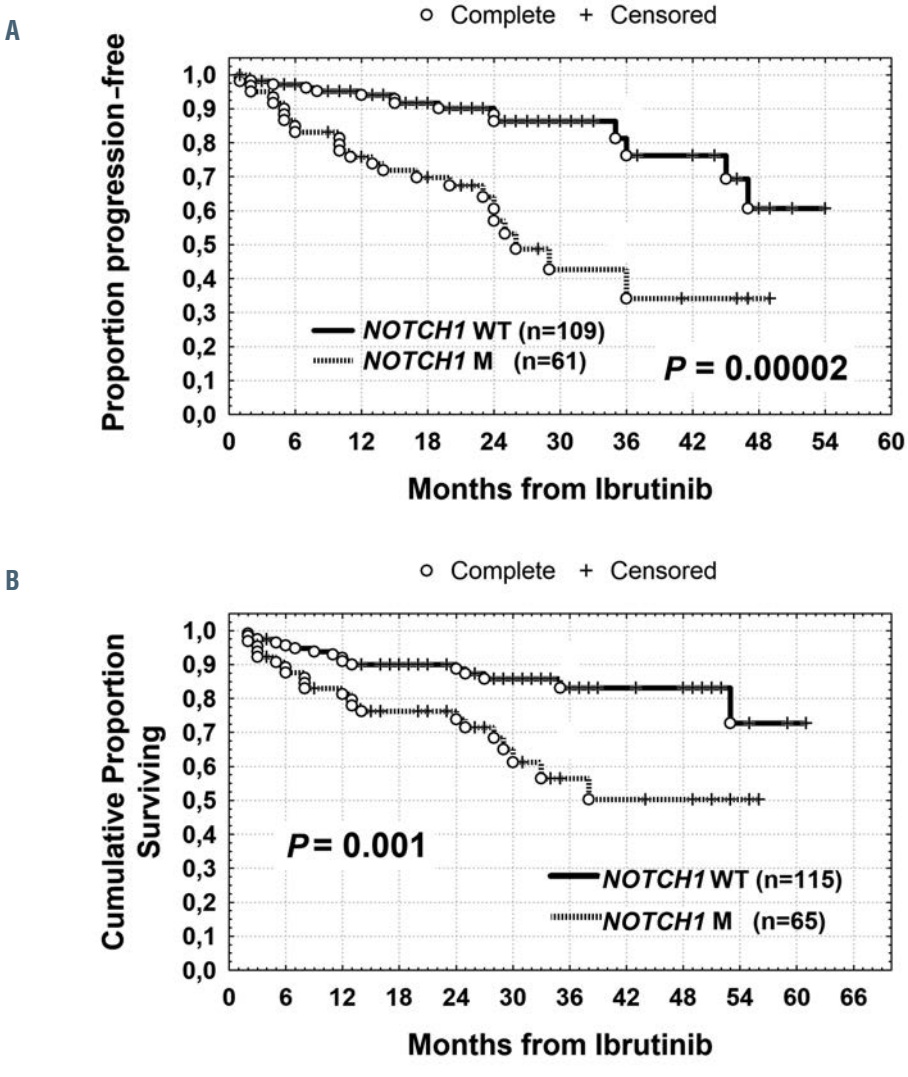

Figure 2. Progression-free survival and overall survival curves based on *NOTCH1*. Kaplan-Meier plot comparing progression-free survival (PFS) (A) and overall survival (OS) (B) based on *NOTCH1*. *NOTCH1* mutated (*NOTCH1 M*) patients experienced both a shorter PFS and OS.

with >2 lines of therapy (*Online Supplementary Figure S5*). On the other hand, the clinical outcome was similar for patients receiving first-line ibrutinib and patients with one previous therapy, probably due to the high incidence of *TP53* mutated patients (24 of 26) in first-line setting (*Online Supplementary Figure S5*).

In CLL, the frequency of *NOTCH1 M* cases between 6-12% if evaluated at presentation, increases to about 15-20% in the context of fludarabine refractory patients.[18,19] The higher frequency of *NOTCH1 M* characterizing our cohort of patients (36%) could be attributed both to the previous lines of chemotherapy and to the very low cut-off (>1%) chosen for *NOTCH1 M*. The adverse clinical outcome of patients with *NOTCH1 M* CLL was confirmed in univariate analysis in several independent cohorts of patients treated with chemo-immunotherapy.[20-24] Since clonal CLL cells accumulate because of prolonged survival due to impairment of apoptosis, the analysis of the BAX/BCL-2 ratio could be a valid tool to provide information on the chemo-sensitivity of CLL cells.[9,10]

We addressed the clinical impact of both *NOTCH1 M*, evaluated by NGS, and BAX/BCL-2 ratio, determined by flow cytometry, in patients with CLL homogeneously treated with ibrutinib, mainly in a (R/R) setting. Determination of both parameters was done prior to starting ibrutinib therapy.

The NGS approach used for *NOTCH1 M* analysis allowed detection of allele frequency as low as 1%, highlighting the presence of subclonal mutations in 32% of total *NOTCH1 M* cases.[1,16,26] Of note, subclonal *NOTCH1 M* (*i.e.*, VAF<10%) had similar prognostic impact as clonal mutations (*Online Supplementary Figures S6 and S7*); consistently a receiver operating characteristic curve analysis confirmed the use of 1% as optimal cut-off (*Online Supplementary Figures S2*). In this context, detection of *NOTCH1 M* by NGS could be viewed as a useful tool for clinical follow-up of patients as well as for minimal residual disease studies, although the latter use remains speculative at the moment. From a biological point of view, we found a significant relationship between *NOTCH1* and some other prognosticators. In particular, a significant correlation between *NOTCH1 M* and higher CD49d or CD38 expressions was

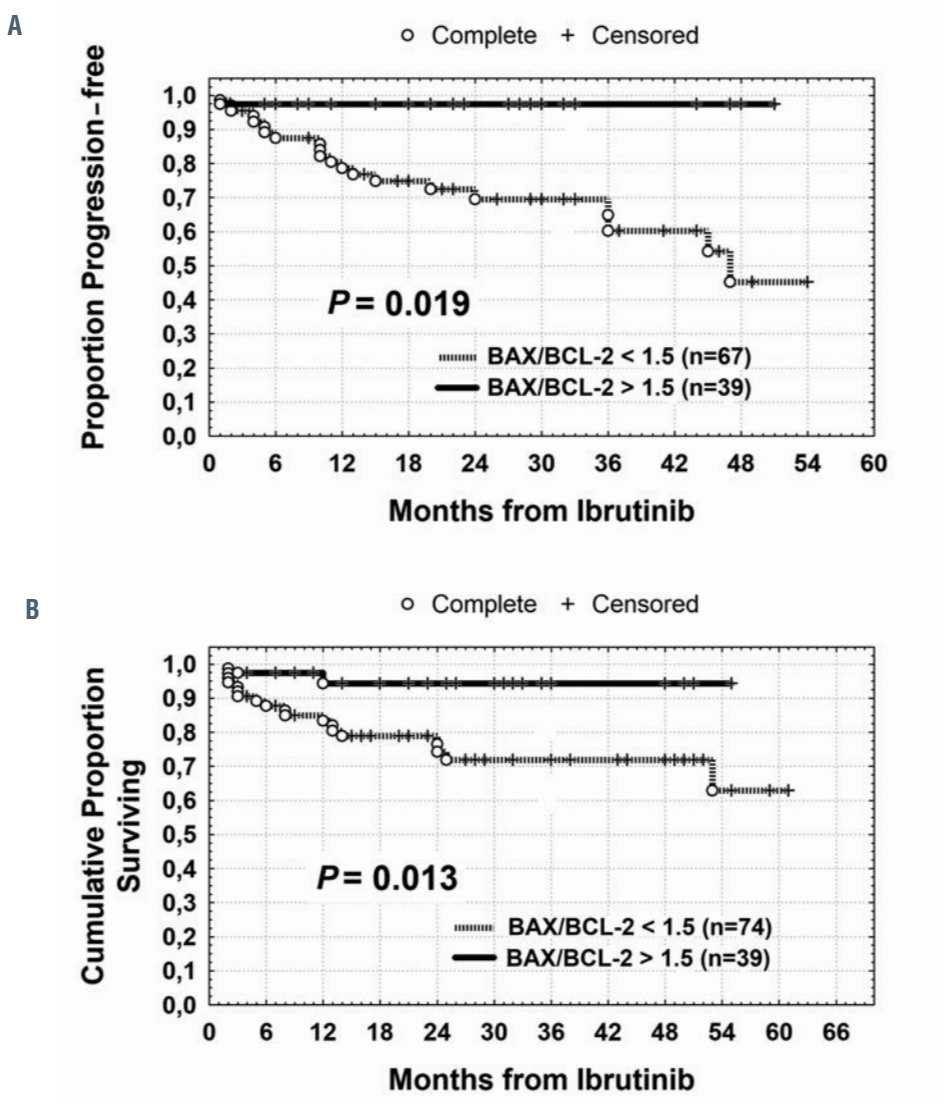

Figure 3. Progression-free survival and overall survival curves based on BAX/BCL-2 ratio within the subset of 113 patients from Rome. Kaplan-Meier plot comparing progression-free survival (PFS) (A) and overall survival (OS) (B) based on the BAX/BCL-2 ratio. Patients with a BAX/BCL-2 ratio <1.5 experienced both shorter PFS and OS.

observed, as well a trend towards an association between *NOTCH1 M* and *IGHV UM* status, in keeping with previous observations by us and others.[27, 21,4,28] Further, co-occurrence of *NOTCH1 M* and *TP53 M* characterized 13% of our patients (23 of 178), a rather high percentage if compared to previous reports where concomitant *NOTCH1* M and *TP53* M, preferentially affecting the same leukemic cells,[29] accounted for 1.2-2.6% of CLL patients.[20,23] This may be due to the high number of pre-treated patients and to the low cut-off chosen by us for *NOTCH1 M* detection. We confirmed that *NOTCH1 M* were strongly correlated with trisomy 12, in line with previous reports describing a high *NOTCH1 M* rate in CLL cases with isolated trisomy 12 and a lower frequency in cases characterized by additional chromosomal abnormalities.[30-32] In particular, a mutation frequency of 41.9% was reported in aggressive trisomy 12 cases, suggesting a pivotal role of *NOTCH1* activation in this group.[33] Moreover, we observed here a lower BAX/BCL-2 ratio in *NOTCH1 M* patients, in keeping with our previous studies showing *NOTCH1*-dependent activation of the NF-kB pathway that may result in the upregulation of target genes, including *BCL-2*.[4]

The strong correlation between lower BAX/BCL-2 ratio and *NOTCH1 M* suggests that the poor prognosis of *NOTCH1 M* patients may be related to the lack of apoptosis, although these observations need further confirmation.

The variability in the degree and kinetics of ibrutinib-induced recirculation lymphocytosis has been highlighted by several studies,[34,35] and was also confirmed in the present study. Here we show that at 3 months on ibrutinib, the typical ibrutinib-induced peak of lymphocytosis is observed in *NOTCH1 WT* patients, but not in *NOTCH1 M* cases. Moreover, even though the analysis of nodal response confirmed an overall significant reduction in organomegaly and lymph node size in most cases at 6 months on ibrutinib, *NOTCH1 M* cases experienced a significant lower nodal response compared to *NOTCH1 WT* cases. These results may be explained by the strong correlation between CD49d overexpression and *NOTCH1 M* (51 of 65 cases), in line with the reported involvement of the *NOTCH1* pathway in the regulation of CD49d expression.[4] Consistently, CD49d associates with nodal presentation and subsequent development of lymphadenopathy in patients with CLL.[36]

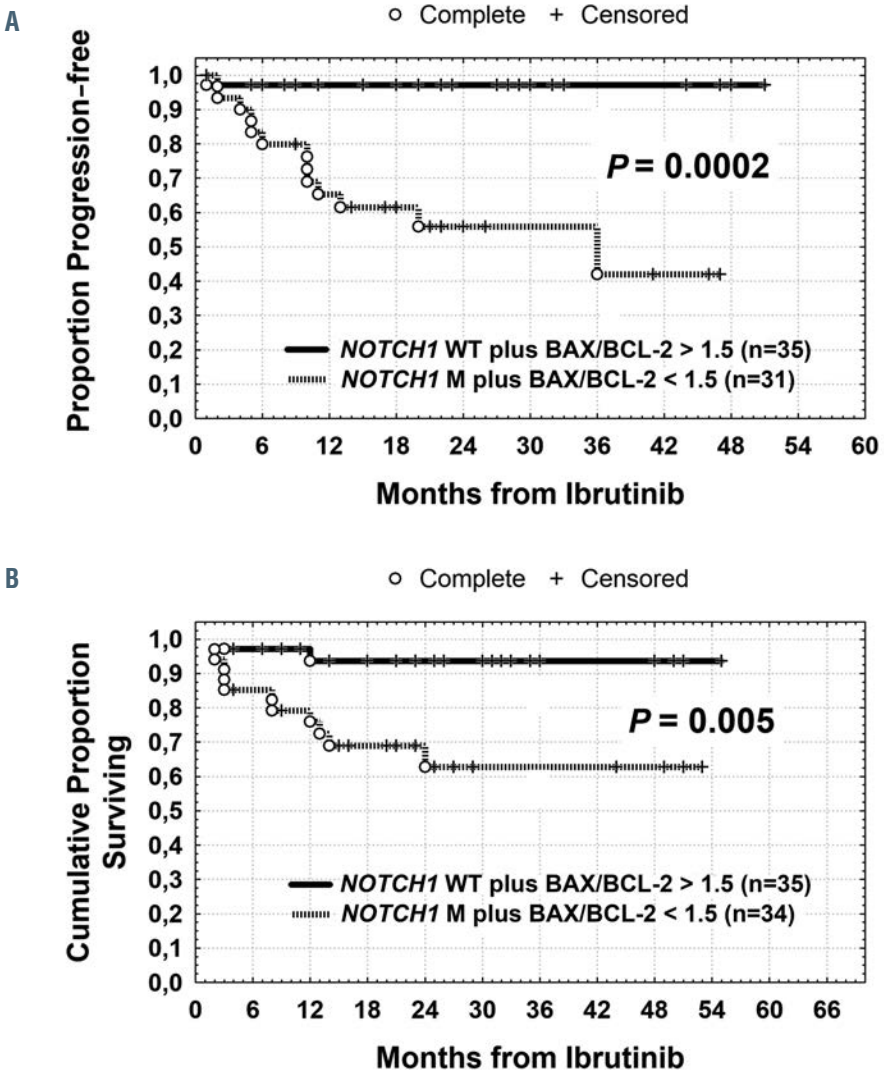

Figure 4. Progression-free survival and overall survival curves in relation to combined BAX/BCL-2 ratio and *NOTCH1*. Progression-free survival (PFS) and overall survival (OS) were shorter within the *NOTCH1* mutated (*NOTCH1 M*) plus BAX/BCL-2 <1.5 subgroup (A-B), showing additive prognostic properties.

Moreover, CD49d expression identifies cases with reduced lymphocytosis and inferior nodal response upon ibrutinib treatment, suggesting the retention of CD49d-expressing cells in tissue sites via activated VLA-4.[7]

Consistently with the high frequency of pre-treated patients in our cohort (154 of 180), the OS values at 2 and 4 years (84% and 71% respectively), were similar to those reported for the phase III RESONATE study in patients with previously treated CLL/SLL.[37]

We have recently reported that *NOTCH1 M* identify a subgroup of patients with CLL with worse prognosis in the setting of a rituximab-based induction and consolidation treatment.[38] Here, we described a negative prognostic impact of *NOTCH1 M* also in the ibrutinib setting. Our findings differ from those resulting from the extended follow up from the RESONATE study of relapsed/refractory CLL, where the presence of *NOTCH1 M* did not negatively affect the efficacy of ibrutinib on disease progression outcomes.[37] This difference can be explained by the very low cut-off (>1%) chosen for *NOTCH1 M* in our study, although for the validation of these findings additional independent cohorts are needed.

The here reported capacity of BAX/BCL-2 index to identify patients with a different response to ibrutinib could be of interest in the light of the treatments protocols associating B-cell receptor inhibitors and BH3 mimetics such as venetoclax.[39]

Moreover, the additive negative prognostic value of *NOTCH1 M* and low BAX/BCL-2 ratio described by us, further support the rationale to improve the efficacy of ibrutinib by using the BCL-2 inhibitor venetoclax in patients with *NOTCH1* mutated CLL.[10] Interestingly, an additive prognostic impact of the combination of BAX/BCL-2 and *NOTCH1 M* in the setting of chemo-immunotherapy was also reported by us.[10]

Several independent cohorts of patients confirmed the adverse clinical outcome of *NOTCH1 M* with CLL in univariate analysis,[20-23,40] although conflicting results are reported about its independent prognostic effect. In particular, *NOTCH1 M* did not retain independent significance as a predictor of time-to-first treatment in one of the largest series of patients with CLL,[41] while in another study it emerged as an independent predictor of shorter survival, along with *TP53* abnormalities.[42] Here *NOTCH1 M* were confirmed to be an independent prognostic factor together with previous lines of therapy and *TP53* both with respect to PFS and OS. The apparent higher prognostic impact of *NOTCH1 M* compared to *TP53* mutation, as emerged in our multivariable analysis, may be explained by the greater number of *TP53* mutated cases treated first line with ibrutinib, hence with a better prognosis than *NOTCH1 M* cases that were more frequently treated with ibrutinib in second or further lines of therapy.

The current use of B-cell receptor and BCL-2 inhibitors led to high-rate improvement of outcome in CLL. However, several issues remain, resulting in resistance/progression thus limiting the eradication of the tumour. The growing evidence for a critical role of the *NOTCH1* pathway in CLL makes this cancer gene a target to design tailored treatments for this peculiar subset through specific *NOTCH1*-targeted therapies. In this context, γ-secretase inhibitors are the most extensively explored anti-*NOTCH1* molecules and their combination with fludarabine demonstrated antitumour effects in primary CLL with *NOTCH1 M*.[43] Noteworthy, a humanized antibody targeting *NOTCH1* (clinicaltrials gov. Identifier: OMP-52M51) entered phase I trial in relapsed/refractory lymphoid malignancies.[44] However, to date, the future treatment of CLL with *NOTCH1 M* relies on the association of small molecule inhibitors targeting both the BCR pathway and the anti-apoptotic BCL-2 protein.

Disclosures

No conflicts of interest to disclose.

Contributions

GDP and VG designed the study, interpreted data, performed statistical analysis, wrote the manuscript and gave final approval of the manuscript; AB and AZ contributed to study design and data interpretation and to write the manuscript; LL, AC and MIDP contributed to interpret the data and to write the manuscript; AZ, FMR and GDP obtained flow cytometric data; FMR performed FISH cytogenetic analysis; VG, FP and RB investigated IGHV, NOTCH1 and TP53 mutations; FB, SA, GG, AV contributed to study design and data interpretation; II, MP, PdF, MC recruited the patients and collected clinical data.

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