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ORIGINAL ARTICLE

Prediction of optimal cytogenetic responses at 6 and 12 months in patients with chronic myeloid leukemia in chronic phase treated with imatinib

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Summary

Purpose: *Imatinib mesylate transformed the treatment and paradigms of chronic myeloid leukemia. European LeukemiaNet (ELN) group has defined specific treatment milestones with an optimal outcome to be achieved in patients.*

Methods: *In a retrospective cohort study, we evaluated the impact of clinical and biological variables on achieving an optimal response at 6 and 12 months according to ELN recommendations. We included 106 patients with chronic phase chronic myeloid leukemia (CML) with appropriate bone marrow aspirate and biopsy for immunohistochemistry.*

Results: *The number of white blood cells (WBC), the percentage of peripheral blast, the values of Sokal and ELTS scores and the percentage of Ki-67+ cells in the bone marrow predicted a complete cytogenetic response (CCyR) at 6 months, but only WBC and EUTOS score predicts CCyR at 12 months. We found that Sokal score below 0.775 was very sensitive to achieve of CCyR at 6 months (m) and that all patients with a value <0.550 achieved CCyR-6m. Patients with a low percentage of blast in the peripheral blood (≤1.5%) or in the bone marrow (≤5%) together with lower WBC (≤100×10^9/L) were likely to have significantly higher CCyR rates at 6 and 12 months respectively. Also, patients with a higher number of Ki67+ cells in the leukemic areas of the bone marrow had a significantly better outcome. Unfortunately, our investigation did not reveal that bone marrow fibrosis (MF grade), microvascular density, percentage of CD34+, CD61+ or PTCH1+ cells could have any effect on achievement of CCyR at 6 or 12 months.*

Conclusion: *Our investigation has shown that only a few biological characteristics in patients with CML can predict the optimal treatment outcome after imatinib.*

Key words: *chronic myeloid leukemia, imatinib treatment, optimal outcome, European LeukemiaNet recommendations, prognostic scores*

Introduction

The introduction of imatinib mesylate in the treatment of chronic myeloid leukemia (CML) transformed the overall outcome in all patients with CML. The first goal of imatinib treatment was to postpone the transformation of CML to acute leukemia and to decrease the number of CML-related deaths, but with the experience gathered with an increasing number of treated patients worldwide, it has become apparent that some patients may achieve a deep molecular response, with a possibility of treatment free remission [1,2].

Since the publication of the first European LeukemiaNet (ELN) recommendation in 2006, it was apparent that achieving milestones in treatment

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has had a major impact on patient outcomes and clinical decision making, leading to three more updates (in 2009, 2013 and 2020) [1,3,4].

In the initial assessment of each CML patient, it is very important to estimate the initial biology of CML, and several different scoring systems are used to predict overall outcome and survival (Sokal, Euro and ELTS-EUTOS long-term survival score) [1,2] or treatment outcome (EUTOS score) [1,2,4], as some patients may need more active treatment, such as the initial use of potent second generation tyrosine kinase inhibitors (TKIs). These cores are mainly based on various biological variables that describe the leukemia itself, but also provide an easy system for the patient evaluation. Therefore, this approach allows the adjustment of individual treatment in accordance with the risk assessment and the stage of disease.

Bone marrow assessment is still the basic diagnostic procedure (both aspiration and needle core biopsy) that provides the necessary information about the CML phase and cytogenetic evaluation needed to establish the diagnosis of CML [1,2]. Some prognostic parameters can be evaluated from the bone marrow itself, such as blast percentage (hence CML phase), bone marrow fibrosis (especially increased) [5-8], but in recent ELN recommendations bone marrow biopsy is not mandatory [1].

During treatment, early molecular response (real time PCR, RQ-$PCR_{bcr\text{-}abl/abl}$ transcript ratio at 3 months <10% or BCR-ABL kinetics in the first 3 months) is a valuable tool for estimation of the overall response later [1,2,9]. Recently, several groups have reported that the expression of patched homologous gene 1 (PTCH1) in a real time PCR assay at diagnosis may have prognostic information about imatinib failure [10,11].

ELN recommendations define several time-dependent milestones to be achieved during the initial treatment of CML. Among them, crucial ones are the complete cytogenetic response at 6 and 12 months, which defines the optimal ELN responses in all recommendations [2-4]. To assess the possibility of predicting the achievement of these two outcomes in patients treated with imatinib, we evaluated several biological parameters that describe CML. The analysis included common clinical parameters such as complete blood counts, blast, basophil and eosinophil percentage in peripheral blood, spleen size (cm below the left costal margin [LCM]) and the corresponding prognostic scoring systems (Sokal, Euro, EUTOS and ELTS) [1,2]. We also evaluated bone marrow findings, including blast percentage on morphology (in bone marrow aspirate), blast percentage estimated as CD34+ cells on immunostaining, initial bone marrow fibrosis, level of dysmegakaryopoiesis (CD61+ megakaryocyte and micromegakaryocyte counts), microvascular density (MVD), the degree of leukemia proliferation described as the percentage of Ki-67+ positive cells and also as the number of cells expressing the PTCH1 protein on immunohistochemistry.

Methods

We retrospectively analyzed 106 consecutive patients with chronic phase CML, treated from 2006 to 2012, with an available high quality biopsy of the bone marrow in the sample repository of the Hematopathology Service at the Clinic of Hematology of the University Clinical Center of Serbia, Belgrade. Patients with an accelerated and blast phase in the diagnosis were excluded from the study. All patients were initially treated with branded imatinib mesylate (Gleevec®, Novartis, Switzerland) in order to compare the data with those previously published. All patients also had a full cytogenetic evaluation at diagnosis with a confirmed karyotype (Philadelphia chromosome or its variants) and almost all patients had typical bcr-abl mutation confirmed by PCR testing [12]. All patients were treated according to the locally adopted ELN 2009 recommendations [3,13], which means that the initial treatment was imatinib 400mg daily, with a change in therapy (escalation of imatinib or nilotinib) in patients with imatinib failure at 6 months only in non-responders, and at 12 months in all non-optimal responders according to ELN 2009 recommendations. Therefore, only 4 patients had changes in treatment (imatinib dose escalation or nilotinib) before 12 months of treatment.

All patients were followed and monitored according to well established recommendations [3,4,14].

All biological specimens (bone marrow aspirate and bone marrow core biopsy) were taken as part of regular diagnostic procedures with the prior consent of the patient. Further retrospective evaluation was performed in accordance with the Declaration of Helsinki and after the approval of the Board of Clinic of Hematology UCC of Serbia and the Ethics Board of the Faculty of Medicine, University of Belgrade.

Bone marrow aspirate slides were stained according to May Grünwald Giemsa staining method with commercial stain (Merck® Darmstadt, Germany). Bone marrow biopsy processing included standardized methods for tissue fixation (10% buffered formalin) and further paraffin embedding process with previous decalcination. The 4 μm thick tissue sections were dehydrated and deparaffinized according to standard procedures, stained with haematoxylin and eosin, Giemsa and Gordon Sweet, and examined by light microscopy (Leica® DM2500 microscope).

Immunohistochemistry (IHC) was performed on decalcified and paraffin embedded bone marrow slides by applying the following monoclonal antibodies: CD34 (clone QBEnd, DAKO®, Denmark), CD61 (clone Y1/51, DAKO®, Denmark), Ki-67 (clone MIB-1, DAKO®, Denmark), PTCH1 (PA1-46222 Thermo Scientific®, Finland), according to the manufacturer's recommendations. Pre-treatment antigen retrieval by microwave heating in citrate buffer (pH6) was

performed according to the manufacturer's instructions and applicable laboratory protocol. Secondary antibody and visualization system was streptavidin-biotin DAB kit (LSAB2, Dako®, Denmark).

Morphological analysis (blast percentage) was performed by counting morphologically evident blasts on 200 cells on blood smears and at least 500 nucleated cells on bone marrow smears. Estimation of the number (percentage) of IHC positive cells (for CD34+ blasts, Ki-67+ and PTCH1+ myeloid cells and CD61+ megakaryocytes) was performed in hypercellular bone marrow areas with frank myeloid tissue by counting positive cells on 10 high power fields (HPF, x400) expressed as percentage [5,8].

After IHC visualization by CD34+ antibody staining, microvascular density (MVD) was performed by counting the vascular loops within hot spots at 10 HPF (x400) expressed as the median number per HPF [15] similarly to the investigation performed in the past in our laboratory [16]. According to the manufacturer's documentation, this HPF area was 0.307 mm2.

Bone marrow fibrosis was evaluated after silver impregnation (Gordon Sweet) according to semiquantitative and morphometric findings and WHO proposals as MF0 to MF3 [17,18].

Statistics

Statistical analyses were performed on IBM SPSS® 17 software (IBM USA), and Statistica® v13 (TIBCO software, USA) platforms using methods of descriptive statistics, nonparametric analysis such as Chi square and Fisher test, Mann-Whitney U test and also receiver operating characteristics (ROC) analysis. Multivariate logistic regression was applied in the analysis of the relationship between the analyzed variables and long-term treatment stability. Exact p values were calculated within the appropriate tests, and p values less than 0.05 were considered significant.

Results

Out study included 106 patients with a diagnosis of chronic phase CML, who had not been previously treated before bone marrow assessment. Their mean age was 47 years (19-74). Basic clinical and hematological data are shown in Tables 1 and 2. All patients were treated with imatinib as the CML active drug, and hydroxyurea was used shortly before switching to imatinib.

Table 1. Initial characteristics of patients with chronic phase myeloid leukemia

Variables	*Median*	*Range*
Age (y)	47	19-74
Hemoglobin (g/L)	121.5	78-173
WBC (x109/L)	98.5	11-553
WBC ≥100x109/L (%)	52	49
Platelets (x109/L)	410.5	145-2212
<450x109/L (patients, %)	61	57.5
>600x109/L (patients, %)	33	31
Blasts (peripheral blood, %)	1	0-14
Basophils (peripheral blood, %)	3	0-13
Eosinophils (peripheral blood, %)	1	0-9
Blasts (bone marrow, %)	4	0-11
1-5	65.1	
6-11	34.9	

Table 2. Initial characteristics of patients with chronic phase myeloid leukemia (scores)

Variables			
Spleen (cm below LCM)	*absent*	*up to 5cm*	*> 5cm*
Patients (number, %)	31 (29.2)	41 (38.7)	34 (32.1)
Bone marrow fibrosis	MF-1	MF-2	MF-3
Patients (number, %)	52 (49.1)	48 (45.3)	6 (5.7)
Prognostic score (number, %)	*low*	*int*	*high*
Sokal	37 (34.9)	44 (41.5)	25 (23.6)
Euro	48 (45.3)	49 (46.2)	9 (8.5)
EUTOS	94 (88.7)		12 (11.3)
ELTS	70 (66.0)	27 (25.5)	9 (8.5)

In our group, a complete cytogenetic response to 6 months of treatment (CCyR-6m) was achieved in 76 of 106 patients (71.7%), and a complete cytogenetic response to 12 months of treatment (CCyR-12m) in 82 of 106 patients (77.4%), but as 4 patients changed treatment after failure at 6 months, the actual CCyR at 12 months was 80.3%. During initial treatment and follow up, of the initial 106 patients, 67 (63.3%) remained stable on the standard dose of imatinib (400mg), while 39 patients (36.7%) had to change the dose of imatinib or switch to nilotinib due to suboptimal response or loss of previously achieved response to treatment.

Detailed analysis of clinical, hematological and selected biological variables in our study, as well as their relationship to the main outcomes, CCyR-6m and CCyR-12m as optimal responses according to ELN recommendations 2013 and 2009, respectively, are presented in Tables 3 and 4 and Figures 1 and 2.

Our findings revealed that white blood cell count (WBC) and blast percentage in peripheral blood were significantly lower in patients achieved CCyR at 6 and 12 months in comparison to non-responders (Mann-Whitney U test $p<0.05$). These two variables were also significantly lower in patients with a long-term stable response to imatinib.

Also, patients with complete cytogenetic response at 6 and 12 months had significantly lower absolute values of all four prognostic scores for Sokal and ELTS scores for response after 6 months

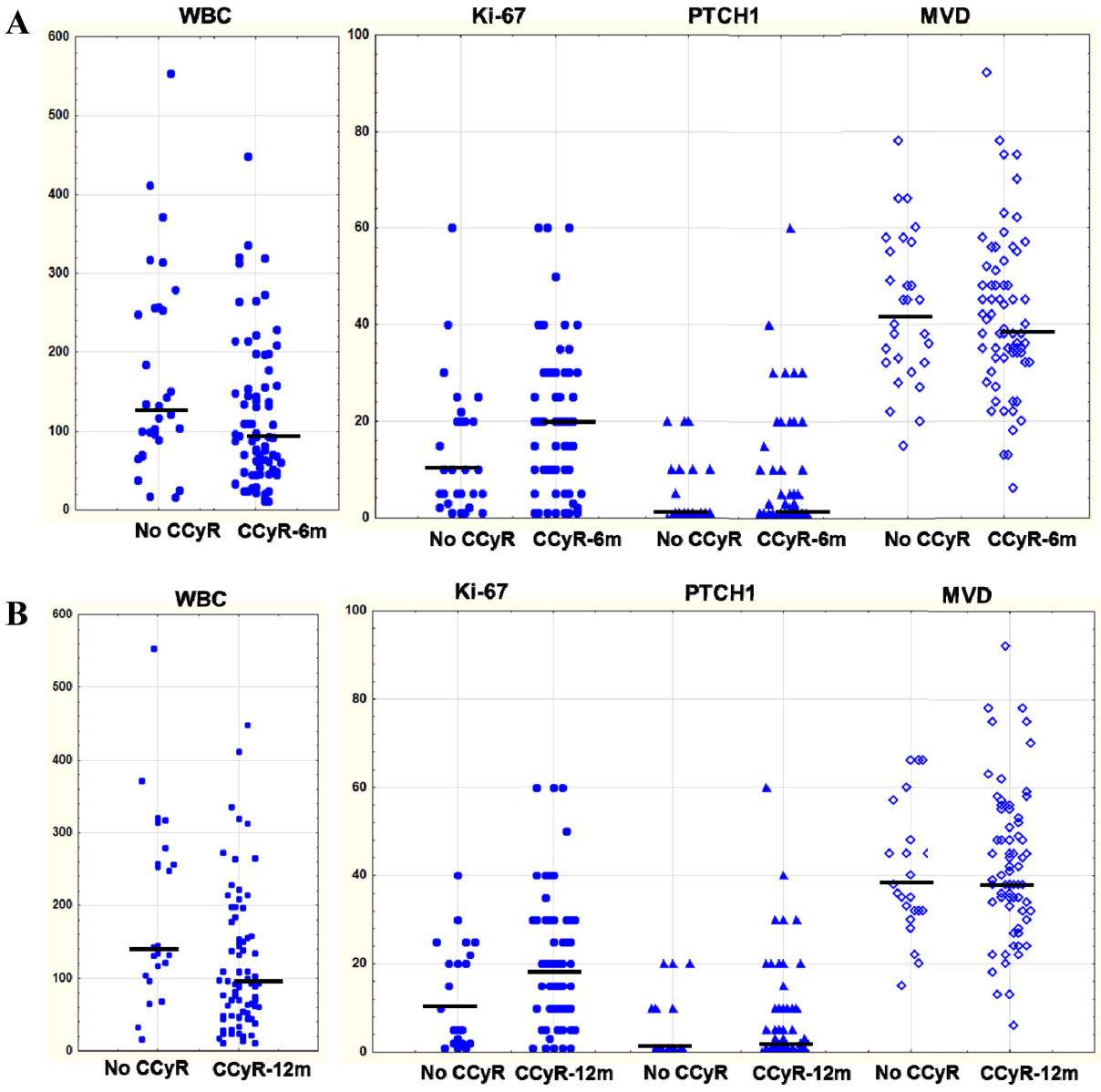

Figure 1. Distribution of values for WBC count (x10^9/L), Ki67+ (%), PTCH1+ (%) and MVD (mean/HPF) according to CCyR at 6 months (scaterplot **A**) and CCyR at 12 months (scaterplot **B**). Bars show medians. Statistical significances are presented on Table 3.

and for EUTOS score for response after 12 months. We could not show statistical significance that patients with high-risk scores had a poorer outcome of imatinib treatment, but the number of high-risk patients was relatively small, especially for EUTOS and ELTS scores (Tables 2 and 3).

Analyzing other biological variables associated with CML, especially those detected by immunohistochemistry, we were not able to show that patients with CCyR at 6 and 12 months had significantly different blast counts and percentages of CD34+ and CD61+ cells in the bone marrow in comparison with non-responders (Table 3 and Figure 2). In contrast, we showed that patients with CCyR-6m had a significantly higher percentage of Ki-67+ cells within the bone marrow, but not for CCyR-12m (Table 3 and Figure 1). A similar trend, but without statistical significance, was observed in the expression of PTCH1+ cells where patients with CCyR had higher values compared to non-responders. For the two later variables, we also found higher values in patients with a stable response to imatinib (for Ki-67, p=0.03, and for PTCH1 p=0.081) (Table 3 and Figure 1).

According to these findings, we performed a ROC analysis to evaluate the possibility of specific cut-off values for variables that showed a statistical difference on the Mann-Whitney U test. ROC analysis showed the cut-off value for WBC count for the outcome of CCyR-6m was 99.5×10^9/L with a sensi-

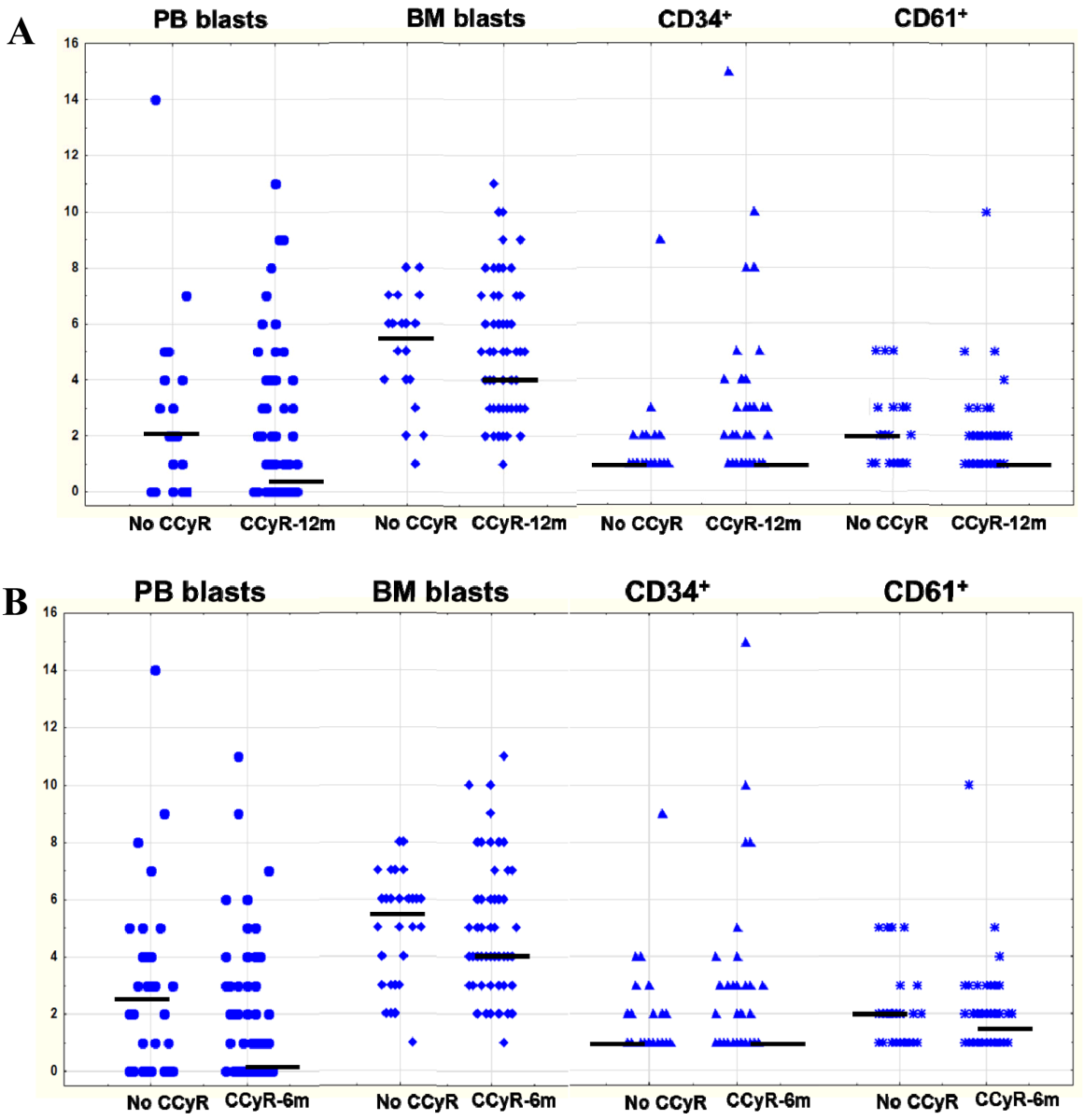

Figure 2. Distribution of values for blast count in peripheral blood and bone marrow, CD34+ and CD61+ cells (%) according to CCyR at 6 months (scaterplot **A**) and CCyR at 12 months (scaterplot **B**). Bars show medians. Statistical significances are presented on Table 3.

tivity of 0.667 and a specificity of 0.579. Similarly, for CCyR at the 12 month cut-off was 110×10^9/L with a sensitivity of 0.708 and a specificity of 0.659. Therefore, we concluded that the previously published critical value of the WBC count of 100×10^9/L is absolutely suitable for further evaluation. ROC analysis for the peripheral blast percentage revealed that the cut-off value for CCyR-6m was 1.5% with a sensitivity 0.60 and specificity of 0.618 and the same value was calculated for the outcome of CCyR-12m with a sensitivity of 0.667 and a specificity of 0.622. In further evaluation of outcomes and relationships, we chose 1.5% as the cut-off value for the peripheral blast count. Finally, ROC analysis for Ki-67+ cells showed that the cut-off value for CCyR-6m was 7.5% with a sensitivity of 0.763 and a specificity of 0.467, so we subsequently decided to use this value for outcome analysis at both 6 and 12 months.

We failed to demonstrate that any current risk stratification system in this series can predict cytogenetic outcomes after 6 and 12 months (Table 4).

Table 3. Differences between analyzed variables in patients with or without complete cytogenetic response at 6 and 12 months of treatment. Statistical significance (Mann Whitney U test) and significant values are bolded. Last column, multivariate logistic regression analysis shows the influence of different variables on stable response to imatinib

	No CCyR at 6m median (range)	*CCyR at 6m median (range)*	*p value*	*No CCyR at 12m median (range)*	*CCyR at 12m median (range)*	*p value*	*Stable response to imatinib*
Hb (g/L)	117.5 (84-159)	126 (78-173)	0.115	117.5 (97-159)	123.5 (78-173)	0.691	0.249
WBC (x109/L)	126.5 (16-554)	90 (11-448)	**0.031**	138.5 (16-554)	90.5 (11-448)	**0.004**	**0.030**
Platelets (x109/L)	378 (145-2212)	441 (150-1953)	0.483	379 (145-1345)	415 (150-2212)	0.607	0.702
Blasts in PB (%)	2.5 (0-14)	0.5 (0-11)	**0.019**	2.0 (0-14)	0.5 (0-11)	**0.030**	0.051
Basophils (%)	3 (0-12)	3 (0-13)	0.606	3 (0-12)	3 (0-13)	0.282	0.076
Eosinophils (%)	1 (0-9)	1 (0-6)	0.838	1 (0-6)	1 (0-9)	0.930	0.729
Spleen (cm below LCM)	4 (0-15)	2 (0-30)	0.149	4 (0-15)	1.5 (0-30)	0.090	
EUTOS score	48 (11-118)	29 (0-134)	0.090	55 (11-116)	29 (0-134)	0.014	0.115
Euro score	928 (42-1863)	724.5 (11-2165)	0.084	878.7 (42-1863)	831.7 (11-2165)	0.255	0.280
Sokal score	0.95 (0.54-3.89)	0.83 (0.47-5.34)	**0.019**	0.93 (0.54-3.58)	0.84 (0.47-5.34)	0.155	0.329
ELTS score	1.60 (0.56-3.30)	1.37 (0.49-3.21)	**0.035**	1.58 (0.49-3.30)	1.37 (0.51-3.21)	0.106	0.216
Blast in bone marrow (%)	5.5 (1-8)	4.0 (1-11)	0.219	5.5 (1-8)	4.0 (0-11)	0.176	0.806
CD34+ cells in BM (%)	1.0 (1-9)	1.0 (1-15)	0.938	1.0 (1-9)	1.0 (1-15)	0.623	0.404
CD61+ cells in BM (%)	2.0 (1-5)	1.5 (1-10)	0.527	1.0 (0-5)	2.0 (1-10)	0.631	0.844
PTCH1+ cells in BM (%)	1.0 (0-20)	1.0 (0-60)	0.166	1.0 (0-20)	1.0 (0-60)	0.196	0.081
Ki-67+ cells in BM (%)	10.0 (1-60)	20.0 (1-60)	**0.028**	10.0 (1-40)	17.5 (1-60)	0.099	**0.033**
MVD in BM (mean/HPF)	42.5 (15-78)	38.0 (6-92)	0.405	37.0 (15-66)	38.0 (6-92)	0.637	0.999

Bold numbers show statistical significance

Table 4. Analysis of variables and specific outcomes after nonparametric or ROC analysis

		CCyR-6 months	*Chi square/Fisher test*	*CCyR-12 months*	*statistics (Chi square/Fisher test)*
PB blasts	*All groups*		*x^2, p=0.092*		*x^2, p=0.128*
	0	38/47		41/46	
	0-≤5	33/50		31/42	
	>5	5/9		10/14	
	0-5 vs >5		x^2, p>0.05		non significant
	0 vs ≥1		x^2, p=0.061; p=0.048		x^2, p=0.044; p=0.037
BM blasts	≤5 vs >5	54/69 vs 22/37	x^2, p=0.041; p=0.035	57/69 vs 25/33	n.s
WBC >100	≤100 vs >100	44/54 vs 32/52	x^2, p=0.023; p=0.019	48/52 vs 34/50	x^2, p=0.002; p=0.002
PB blast	≤1.5% vs >1.5%	49/61 vs 27/45	x^2, p=0.021; p=0.019	52/59 vs 30/43	x^2, p=0.020; p=0.020
Ki-67	>7.5% vs ≤7.5%	58/74 vs 18/32	x^2, p=0.020; p=0.020	61/70 vs 21/32	x^2, p=0.011; p=0.013
Sokal	<0.775 vs ≥0.775	31/34 vs 45/72	x^2, p=0.022; p=0.0014	31/34 vs 51/79	x^2, p=0.019; p=0.015
EUTOS	51 vs ≥51	50/65 vs 26/41	non significant	55/63 vs 27/39	x^2, p=0.045; p=0.041

Analyzing the data for Sokal score values after ROC analysis, it was found that there are differences between groups with or without CCyR during 6 months, as ROC analysis found that Sokal score below 0.775 is highly significant for achieving CCyR in 6 months (Chi square p=0.002, Fisher p=0.0013) where 31 of 34 patients achieved CCyR (sensitivity 0.90 and specificity 0.408) (Table 4). Similar discrimination was found for CCyR after 12 months (Table 4, Chi square p=0.019, Fisher 0.015). This threshold was slightly below the classic definition of low risk Sokal score value (less than 0.800). We also observed that all patients with Sokal score less than 0.550 achieved CCyR at 6 months.

Regarding EUTOS score, a ROC analysis was performed to confirm whether the current cut-off of 87 could be replaced by different value more suitable for risk assessment, as a univariate analysis revealed differences in the EUTOS score values for outcome, CCyR-12m (Table 3). The EUTOS score of 51 provides better separation for CCyR-12 months, with a sensitivity of 0.571, and a specificity of 0.667. Thus, we found that 55 of 63 patients (87.3%) with EUTOS value below 51 achieved CCyR-at 12 months, and 27/39 (69.2%) with EUTOS value over 51 (Chi square, p=0.045, Fisher p=0.041) (Table 4).

Certain differences were also found between responders and non-responders in terms of the peripheral blast percentage, the bone marrow blast count and also categorical groups according to the newly defined cut-off values for WBC number and the percentage of Ki67+ cells in bone marrow (Table 4). It was found that patients with a WBC count over 100×10^9/L were less likely to achieve CCyR at 6 and 12 months, respectively (Chi square and Fisher tests, both p<0.01, Table 4). Patients without blasts in the peripheral blood have been shown to be much more likely to achieve CCyR at 6 and 12 months than those with left shifting and blasts in the peripheral differentials. According to the ROC analysis, patients with less than 1.5% blasts in the peripheral differentials were significantly more likely to achieve both cytogenetic remissions (6 and 12 months) and to remain stable on imatinib in the long-term (p=0.051) (Tables 3 and 4). The classic distinction between the blast bone marrow count ≤5% and over 5% also showed that patients with more than 5% blasts were significantly less likely to achieve CCyR-6, but not at 12 months (Table 4).

Contrary to such behaviour of WBC and blast counts, it was found that the expression Ki-67 in the bone marrow measured by IHC had a different trend. Patients with increased myeloid proliferation (e.g. Ki-67+ cells over 7.5% estimated on ROC analysis) were more likely to achieve CCyR at both 6 and 12 months (78 vs 56% at 6 months and 87 vs 66% at 12 months, respectively) (Table 4).

Discussion

Our findings revealed that certain but not all biological variables determined as important in the past continued to have a prognostic effect on imatinib-treated patients. In our group of patients, we found that the overall impact of WBC counts was similar to some other publications, such as the European EUTOS CML registry, where WBC counts were slightly below 100×10^9/L [19-21], and that almost half of patients (49%) had a high WBC count, over $>100\times10^9$/L. On the contrary, some reports have an even higher median WBC count [22,23]. We found that responding patients had significantly fewer WBC than non-responders (Mann-Whitney U test, p=0.031). This finding is also similar to some recent publications [20,23,24], which also found the significance of WBC count in evaluating the cytogenetic response in real world data.

It is also important that there is a statistical significance of the peripheral blast count in responders versus non-responders in both critical treatment time milestones, 6m and 12m (p=0.026 and p=0.038, respectively) when responding patients had lower or absent blasts in differential counts. ROC analysis confirmed that the threshold of 1.5% blast in peripheral blood is critical as a cut-off value in further evaluation and achievement of CCyR during 6 and 12 months. The blast count in the peripheral blood is also important, because patients with absent or low count blasts in the peripheral blood had a stable response to the standard dose of imatinib (p=0.051 on multivariate logistic regression). A similar finding was observed in a large Korean analysis of 286 patients with chronic phase CML [21]. The influence of the peripheral blast count on different outcomes in CML is not new, because the blast count is one of the main prognostic variables in almost all prognostic scoring systems, from Sokal to the new era with ELTS score [1,2].

In contrast, the bone marrow blast count was not consistent with these findings as the Mann U Whitney test did not find a significant difference in the mean number of bone marrow blast count (p>0.05). But when we assigned the blast count in the bone marrow as ≤5% and over 5%, we noticed significant differences in CCyR rate over 6 months as 78.3% (54 out 69 patients) with low blast count achieved CCyR, but only 59.5% (22 of 37 patients) with blasts over 5% (Chi square, p=0.040). This difference was not significant for CCyR at 12 months (p=0.077). This finding is similar to the finding by

Eliot [25] who found that the marrow blast count with a threshold >5% morphologically affected CCyR at 6, but not at 12 months. As CD34+ count usually serves as a measure of blast equivalents in histopathology, we also assessed the influence of CD34+ cells on cytogenetic responses, although we didn't find a strong statistical correlation between morphologically estimated blast number on CD34+ cell smears in IHC bone marrow biopsies (Spearman rank correlation, $p<0.10$). Our further analysis did not reveal an association with CCyR outcomes ($p>0.05$), unlike Eliot [25] who found a difference in CD34+ cells/10 HPF for cytogenetic outcome after 12 months. It should be noted that he did not include only patients with a chronic phase in his series.

Our investigation failed to find a significant difference in the number of CD61+ cells, neither a difference in the degree of bone marrow fibrosis. Several previous reports on imatinib treatment described changes in fibrosis during treatment [6,8] and, moreover, the prognostic significance of severe marrow fibrosis in terms of treatment efficiency [5-7,26]. Our results showed that patients with mild fibrosis, which is common to almost all patients with CML [5,8], had a significantly better CCyR rate after 12 months (89 vs 72%, Table 4). A similar finding was observed in the work of the MD Anderson group [6], but did not confirm their recent report [5] using the same fibrosis grading (WHO).

MVD has also been reported to have impact on treatment response [27] and overall survival and leukemia-free survival [28]. Moreover, imatinib treatment has been shown to significantly affect bone marrow neovascularization during CML treatment [12] and may reverse this process [29]. In our study, we observed that patients with CCyR-6m had a lower MVD than non-responders, but we failed to show any statistical significance, probably due to the very wide variability within the groups.

Estimation of leukemic cell proliferation is also important in the evaluation of disease biology [8,30]. With the introduction of imatinib almost 20 years ago, this drug has been shown to significantly reduce the proliferation of leukemic cells [8]. As other measurements of good response with prediction of molecular and survival outcome with early molecular response have been identified over time [1,2,9,21], the determination of the proliferative cell fraction in CML has lost relevance. There have been few publications analyzing the prognostic significance of Ki-67+ expression in CML. In our study, we found that patients with complete cytogenetic responses at 6 and 12 months had significantly higher Ki-67+ cells, which can be explained by the efficacy of imatinib treatment on the proliferative fraction of leukemic cell. Moreover, in our analysis, we found that a threshold of 7.5% Ki-67+ cells was important for achieving CCyR both during 6 and 12 months of treatment ($p<0.05$).

Recently, Hammersmith and Spanish group [10,11] reported that real time quantitative PCR expression of the PTCH1 gene can select patients with an overall better outcome on imatinib and other TKIs. In the research, we used different technologies, the application of IHC, having in mind that monoclonal antibodies coupled with IHC procedures are much easier to perform on bone marrow biopsy, without sophisticated technology. In our study, we enumerated PTCH1+ cells in respect to cytogenetic remission at 6 and 12 months of treatment and found that there was a nonsignificant trend that patients with higher numbers of PTCH1+ cells within the bone marrow were more likely to have a complete cytogenetic response (see figures). Furthermore, logistic regression analysis showed similar findings towards achieving stable remission at the standard dose of imatinib ($p=0.081$). This is consistent with previous publications where the level of PTCH1 gene activity has been shown to strongly correspond to several long-term outcomes during imatinib therapy, such as CCyR at 12 months (76 vs 54% for low PTCH1 expression) or stability of imatinib response [10,11]. We assume that due to the different sensitivity of the monoclonal antibody/IHC assay and observed variability, we were not able to repeat these results.

From the clinical point of view, we failed to demonstrate that current scoring systems can determine cytogenetic outcomes, except for EUTOS score for CCyR after 12 months. This is not surprising because these systems were designed predominantly to evaluate survival rather than response at any point, although the EUTOS score was designed to evaluate complete cytogenetic response not with 12 but with 18 months of treatment, which is now obsolete [31]. On the contrary, we have shown that ROC-adjusted cut-offs can predict CCyR at 6 months (Sokal score below 0.775 or even lower 0.550) or CCyR at 12 months (EUTOS score below 51).

Our failure to identify some significant differences may be due to a certain bias, probably due to the small number of patients in our study. On the other hand, our group was homogeneous as it originated from a single academic center and included similarly treated patients according to the same rules, which enabled us to make our deductions.

Besides, we believe that imatinib treatment itself, with very high efficacy in achieving all treatment goals, can overcome many of the negative effects of biological diversity in CML patients with chronic phase.

Conclusion

Our investigation analyzed several clinical, hematological, and biological variables of CML and tried to investigate some prognostic impact by finding a suitable model for predicting optimal responses like a complete cytogenetic response at 6 and 12 months. We found that WBC count, the peripheral blast percentage, and the expression of Ki-67+ cells in the bone marrow were strongly associated with the achievement of optimal responses (CCyR at 6 and 12 months). Moreover, our analysis determined that the cut-off values for these variables (WBC $<100\times10^9$/L, peripheral blasts <1.5% and Ki-67+ cells within the bone marrow >7.5%) have positive power to determine optimal outcomes. We also observed that immunohistochemically detected expression of PTCH1 protein may have certain value in predicting treatment outcomes, but it is evident that PCR-based tests are much more potent.

Our investigation also confirmed that bone marrow assessment should continue to be part of the initial evaluation of all CML patients, needed to determine the stage of disease, and some other prognostic indicators. We also believe that analysis of larger cohorts of patients might help to better define some of our findings.

Conflict of interests

The authors declare no conflict of interests.

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