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RESEARCH

Evaluation of leukemic stem cell (CD26 +) in chronic myeloid leukemia patients with different molecular responses and in treatment-free remission

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Abstract

CD26 + leukemic stem cells (LSC) are a specific marker for chronic myeloid leukemia (CML), absent in healthy individuals and other myeloid neoplasms. These cells can contribute to disease resistance, as they are believed to sustain the leukemic clone despite effective tyrosine kinase inhibitor (TKI) therapy. This study analyzed CD26 + LSC and *BCR::ABL1* transcript levels simultaneously using multiparametric flow cytometry and RT-qPCR in 210 chronic-phase patients undergoing TKI therapy and 31 patients in treatment-free remission (TFR). A significant decrease in LSC levels was observed as patients achieved deep molecular response (DMR, $BCR::ABL1^{IS} \leq 0.01\%$) ($\chi^2$, $p < 0.001$). However, 19% (14/73) of DMR patients displayed persistent CD26 + LSC, suggesting a quiescent state without detectable *BCR::ABL1* transcripts. A weak correlation ($r = 0.187$, $p = 0.046$) between LSC/µL absolute number and *BCR::ABL1* transcript levels indicates a limited predictive value between these two variables. In TFR patients, LSC recurrence during follow-up did not correlate with molecular relapse, questioning their clinical relevance in this setting. In conclusion, while CD26 + LSC are frequently observed in patients with poor molecular response, their levels significantly decrease as patients achieve DMR. However, their persistence or recurrence in TFR lacks prognostic value for molecular relapse, indicating that CD26 + LSC are not reliable predictors of outcomes in CML.

Keywords Chronic myeloid leukemia · Deep molecular response · Treatment-free remission · CD26 + leukemic stem cell · Tyrosine kinase inhibitor

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the translocation t(9;22)(q34;q11.2), which generates the BCR::ABL1 fusion protein with constitutive tyrosine kinase (TK) activity [1, 2]. This oncoprotein leads to myeloid proliferation, inhibition of apoptosis and escape from bone marrow (BM) immunological surveillance. The development of TK inhibitors (TKI) such as imatinib, nilotinib, and dasatinib significantly prolonged patients survival [3–5]. Some patients, however, can acquire mutations within the TK domain of *BCR::ABL1* making them unresponsive to therapy [6]. Another important mechanism is the intrinsic resistance of leukemic stem cells (LSC) to TKI. These LSC may persist in the BM even in patients with undetectable molecular disease [7, 8].

Quantitative reverse-transcription PCR (RT-qPCR) is used for the monitoring of CML patients undergoing TKI therapy [9–11]. This highly sensitive methodology allows to evaluate minimal residual disease, being particularly important in deep molecular responses (DMR $\leq 0.01\%$ $BCR::ABL1^{IS}$), which are associated with better long-term outcomes [12, 13]. However, RT-qPCR is unable to detect non-transcriptionally active cells, highlighting the need for new analytical methods that are independent of transcriptional activity.

Isolating LSC from normal hematopoietic stem cells (HSC) can be challenging since both populations exist within the same multipotent progenitor compartment, characterized by the immunophenotype CD45 + /CD34 + /CD38-. LSC quantification in DMR patients is complex due to their low numbers and their potentially undetectable *BCR::ABL1* levels [14, 15]. In 2014, the surface enzyme CD26 (dipeptidylpeptidase IV, DPPIV) was identified as a specific marker of CML LSC (CD26 + LSC), facilitating discrimination from normal HSC or LSCs of other myeloid neoplasms [8, 16].

Treatment-free remission (TFR) is an emerging goal for many CML patients who have achieved a DMR. Data on TFR patients indicate that up to half of them can relapse within 6 to 12 months post-TKI suspension. The reasons for this high relapse rate remain unclear, with several prognostic factors such as treatment duration, depth of molecular response and Sokal risk score being implicated [17]. However, these factors alone cannot fully explain the loss of molecular response, as patients with similar characteristics can exhibit different outcomes post-treatment suspension. Some studies suggest that the LSC persistence, which can remain quiescent independently of *BCR::ABL1* activity, may contribute to relapse [7, 18–20]. Thus, our study aimed to analyze CD26 + LSC levels in CML patients on TKI therapy with different molecular responses and during TFR, assessing the importance of monitoring residual LSC in patients on or off treatment.

Material and methods

Patient and control samples

Peripheral blood (PB) samples were collected from 210 Argentinian chronic phase CML patients, either at diagnosis or on TKI therapy, as well as two groups of TFR patients: a cohort of 16 cases from the AST-Argentina Stop Trial (NCT05926128) [21, 22] and another group of 15 patients in TFR in real-life settings outside the AST protocol. LSC detection was performed using Multiparametric Flow Cytometry (MFC) and, additionally, RT-qPCR for *BCR::ABL1* rearrangement was carried on simultaneously on the same sample. Patients with other blood disorders and healthy donors served as negative controls for CD26 expression. All participants provided written informed consent, which was approved by our institutional ethics committee (Nº: 36/22/CEIANM) in accordance with the Declaration of Helsinki.

Detection of CML LSC by multiparametric flow cytometry

Samples were analyzed within 24hs using a BD FACS CANTO II flow cytometer. Red cell lysis was performed using BD FACS Lysing™ solution (BD Biosciences, USA) diluted 1:10 in deionized water. CD26 + expression was evaluated on a CD45 + /CD34 + /CD38- cell population using an eight-color staining protocol with a stain-lyse-wash procedure. The samples (250µL) were incubated for 10min with an antibody mixture: HLA-DR (V450) clone L243/ CD45 (V500) clone 2D1/ CD38 (FITC) clone HB7/ CD26 (PE) clone L272/ CD34 (Percp cy5.5) clone 8G12/ CD117 (PE cy7) clone 104D2/ CD123 (APC) clone 9F5/ CD3 (APC-H7) clone Sk7. Specifically, CD123 + /HLA-DR- was employed to discriminate basophils from dendritic cells and CD117 was used to assess myeloid differentiation. All antibodies were acquired from BD Biosciences (USA).

Flow cytometry analysis

Samples were analyzed using a 3-laser, 8-color BD FACS Canto™ II flow cytometer using the FACS Diva software version 8.0 (BD Biosciences, USA) to reach a sensitivity of 10^{-5} and acquiring at least 1.0×10^6 total events. Daily setup and adjustments of internal FACS parameters were performed with BD FACS Diva™ CS&T IVD Beads (BD Biosciences, USA) to ensure consistent instrument

performance overtime. Compensation was set up with BD OneFlow™ Setup Beads and BD™ FC Beads 8-color Kit (BD Biosciences, USA). Cells were gated to exclude debris and doublets. The absolute number of CD26 + cells/µL in PB samples was calculated as: *(WBC count/µL) x (%CD34 + /CD38-/CD26 + on CD45 + cells)*, expressed as LSC/µL [18]. Gating was performed as shown in Fig. 1.

RT-qPCR for *BCR::ABL1* rearrangement and molecular response definitions

Total RNA was extracted from 6 mL of PB. Red blood cells were lysed by successive washes with ammonium buffer. The cell pellet was dissolved in Trizol™ (Life Technology, USA) and RNA extraction followed the manufacturer's recommendations. Taqman method was used to perform *BCR::ABL1* RT-qPCR with primers and probes previously

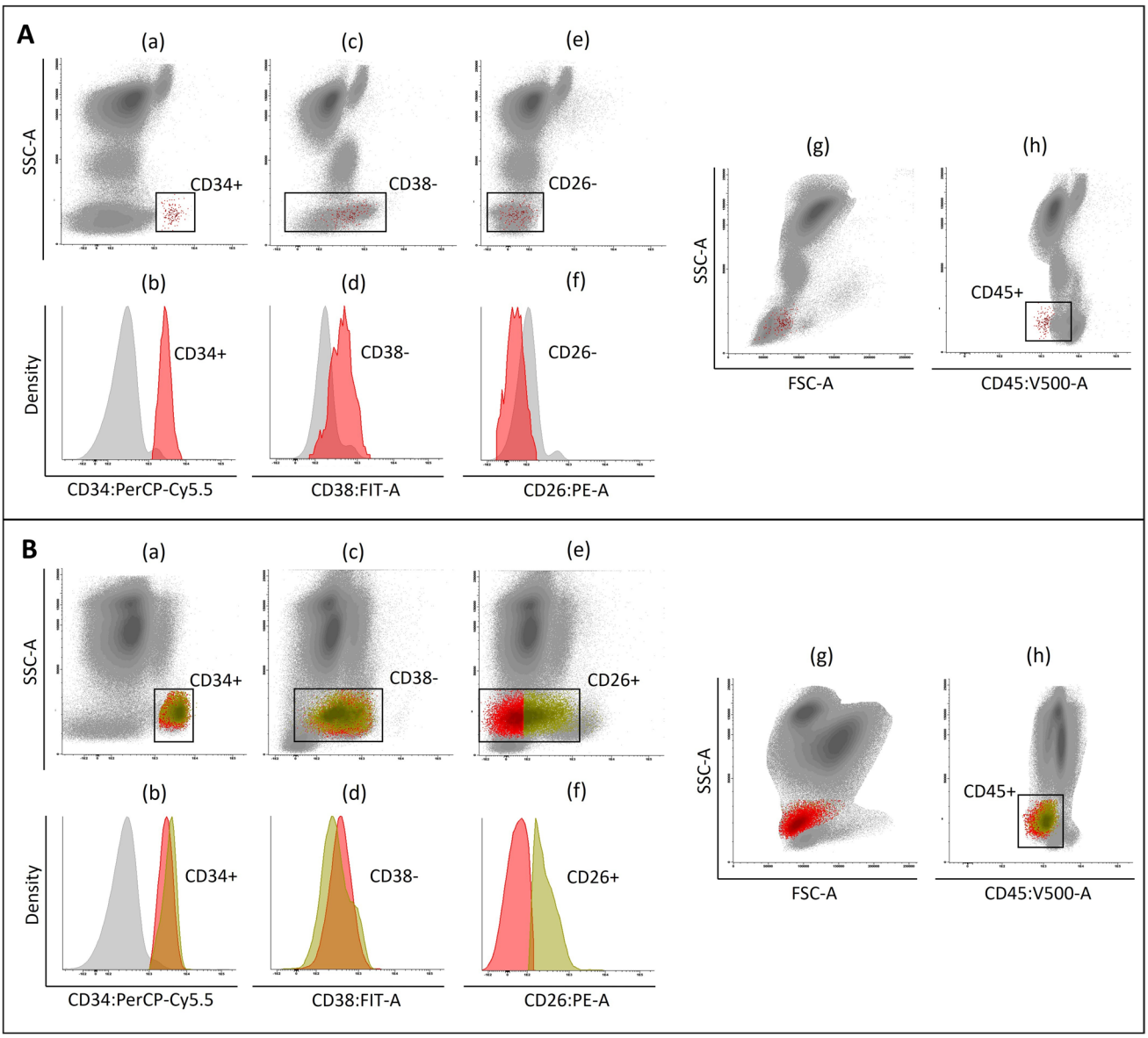

Fig. 1 Flow cytometry analysis of LSC in CML patients. PB from a CML patient in TFR with CD34 + /CD38-/CD26- cells **A** and PB from a CML patient at diagnosis with CD34 + /CD38-/CD26 + cells **B**. Gating was performed on CD34 + viable cells vs SSC-A light properties **a** and **b**. The following graphs are based on the CD34 + population and show consecutive gating of SSC vs CD38 **c** and **d** and SSC vs CD26 populations **e** and **f**. In each panel, gating was performed in the black squares and red dots and histograms represent CD34 + /CD38-/CD26- cells, green dots and histograms represent CD34 + /CD38-/CD26 + cells and grey represents other CD45 + /CD34- hematopoietic populations. According to the characteristics of SSC-A vs FSC-A **g** and SSC-A vs CD45 **h**, sequential gating was performed to exclude debris and doublets

published [23], utilizing a Rotor-Gene 6000 Q (QIAGEN, USA) thermal-cycler. Values are expressed on International Scale (*IS*) levels, with a specific correction factor from our laboratory, based on the 1st WHO International Genetic Reference Panel (NIBSC code: 09/138) *ABL1* was the reference gene employed. This study evaluated the *BCR::ABL1/ABL1* ratio according to *IS*: $BCR::ABL1^{IS} \leq 0.1\%$ indicates Major Molecular Response (MMR, or $MR^{3.0}$). DMR includes $MR^{4.0}$, $MR^{4.5}$, and $MR^{5.0}$, corresponding to: $BCR::ABL1^{IS} \leq 0.01\%$ (≥ 4 log reduction), $\leq 0.0032\%$ (≥ 4.5 log reduction) and $\leq 0.001\%$ (≥ 5.0 log reduction) levels from the standardized baseline, respectively [9, 12, 24].

Statistical analysis

Statistical analyses were performed using SPSS version 27 (IBM Corp, USA). Mann–Whitney U (MW) and Kruskal–Wallis (KW) tests were applied to evaluate non-parametric variables between independent groups, while Chi-Square or Fisher's exact tests were used to assess positive cases proportions in each molecular category. The Kendall rank correlation coefficient was used to detect associations between non-parametric continuous variables, after applying a logarithmic transformation. All tests were two-sided, with $p < 0.05$ considered statistically significant and $p < 0.001$ highly significant.

Results

CD26 + LSC dynamics and molecular response in TKI-Treated CML patients

The study included 210 chronic phase CML patients on TKI therapy. The on-treatment cohort comprised 6 newly diagnosed cases and 204 cases treated with imatinib ($n = 101$), nilotinib ($n = 59$), or dasatinib ($n = 44$) for treatment median durations of 4.8, 3.3, and 1.5 years, respectively. Demographic data and molecular outcomes for each molecular category are summarized in Table 1.

The study of CD26 + LSC and the molecular response was conducted on random samples during the follow-up period (Table 2). The CD26- and CD26 + stem cell populations were analyzed by MFC, as shown in Fig. 1. CD34 + /CD38-/CD26 + cells were detected in 67 (31.9%) of the 210 analyzed patients, with a median of 0.29 cells/µL (range: 0.001–890.010). As expected, all patients at diagnosis exhibited a *BCR::ABL1* ratio greater than 10%, with only 1/6 (16.6%) lacking detectable CD26 + LSC. When analyzing the presence of CD26 + LSC across the different molecular response categories according to the *IS*, a significant decrease in the proportion of cases with CD26 + LSC was observed alongside the improvement of the molecular response ($\chi 2$, $p < 0.001$) (Fig. 2A).

Table 1 Characteristics of patients under TKI treatment

Analyzed population ($n = 210$)		
Age (years)	Median (Range)	51 (18–90)
Sex n (%)	Male/Female	118 (56.2)/92 (43.8)
Treatment n, Median duration (years) (range)	Newly diagnosed	6
	Imatinib	101, 4.8 (0.1–21.1)
	Nilotinib	59, 3.3 (0.8–15.3)
	Dasatinib	44, 1.5 (0.1–14.5)
Molecular Response n (%)	DMR ($\leq 0.01\%$)	73 (35)
	MMR ($\leq 0.1\%$)	54 (26)
	Minor MR ($> 0.1\%$ $BCR::ABL1^{IS}$)	38 (18)
	Min MR ($> 1\%$ $BCR::ABL1^{IS}$)	21 (10)
	Null MR ($\geq 10\%$ $BCR::ABL1^{IS}$)	24 (11)

MR, Molecular Response; DMR, Deep MR (includes $MR^{4.0}$, $MR^{4.5}$, $MR^{5.0}$); MMR, Major MR; Min, Minimal

The same pattern was observed when analyzing CD26 + LSC in relation to *BCR::ABL1/ABL1* ratio, with a significant decrease in CD26 + LSC number as transcript levels diminished (MW, $p < 0.001$) (Fig. 2B), showing an association between LSC burden reduction and the improvement on molecular response. When comparing the frequency of CD26 + LSC across molecular categories for the three different treatments, no significant differences were identified (Table 2). However, among patients who achieved a DMR, nilotinib showed a more pronounced reduction in CD26 + LSC compared to imatinib and dasatinib ($\chi 2$, $p < 0.001$) (Table 2). This greater reduction observed with this TKI was not influenced by treatment duration, as no significant differences were found when comparing the length of treatment between nilotinib and either imatinib (MW, $p = 0.127$) or dasatinib (MW, $p = 0.063$).

For patients in DMR, the analyzed cohort included 73 cases: 32 were in MR4.0, 37 in MR4.5 and 4 in MR5.0 (Md: 58.6 years, range: 24.7–85.1). Treatment distribution was as follows: 56.2% on imatinib (41/73), 24.7% on nilotinib (18/73) and 19.2% on dasatinib (14/73), with median durations of 8.2 years (range: 0.3–21.1), 5.8 years (range: 2.4–11.1), and 2.9 years (range: 0.2–14.5), respectively for each TKI (Table 2). Despite the depth of the molecular response achieved, 18.8% (6/32) of patients in MR4.0 and 19.5% (8/41) in $\geq$ MR4.5 showed persistence of CD26 + LSC, suggesting a potentially quiescent state for these cells.

To determine whether *BCR::ABL1* transcripts levels are associated with the absolute number of CD26 + cells/µL, we performed a Kendall rank correlation test. Although

Table 2 CD26 + LSC frequency according to molecular category and TKI treatment[a]

	Total $n=210$		Imatinib $n=101$		Nilotinib $n=59$		Dasatinib $n=44$		
Molecular Response	CD26- $n=143$	CD26+ $n=67$	CD26- $n=68$	CD26+ $n=33$	CD26- $n=45$	CD26+ $n=14$	CD26- $n=29$	CD26+ $n=15$	χ^2 p value
Null MR[b] (%)	5 (20.8)	19 (79.2)	1 (12.5)	7 (87.5)	1 (16.7)	5 (83.3)	2 (50.0)	2 (50.0)	0.312
Min MR (%)	9 (42.9)	12 (57.1)	4 (57.1)	3 (42.9)	3 (42.9)	4 (57.1)	2 (28.6)	5 (71.4)	0.558
Minor MR (%)	28 (73.7)	10 (26.3)	12 (66.7)	6 (33.3)	7 (77.8)	2 (22.2)	9 (81.8)	2 (18.2)	0.634
Major MR (%)	42 (77.8)	12 (22.2)	21 (77.8)	6 (22.2)	16 (84.2)	3 (15.8)	5 (62.5)	3 (37.5)	0.464
$MR^{4.0}$ (%)	26 (81.3)	6 (18.8)	15 (75.0)	5 (25.0)	6 (100)	0	5 (83.3)	1 (16.7)	0.384
$\geq MR^{4.5}$ (%)	33 (80.5)	8 (19.5)	15 (71.4)	6 (28.6)	12 (100)	0	6 (75.0)	2 (25.0)	0.125
χ^2 p value	**< 0.001**	0.021	**< 0.001**	0.208					

Bold Values represent highly significant p-values (<0.001)

MR, Molecular Response; Min, Minimal; X^2, Chi square distribution

[a]Percentages were calculated by molecular category and inhibitor

[b]Six patients were analyzed at diagnosis; all had Null MR. Only 1/6 (16.7%) had undetectable CD26 + LSC

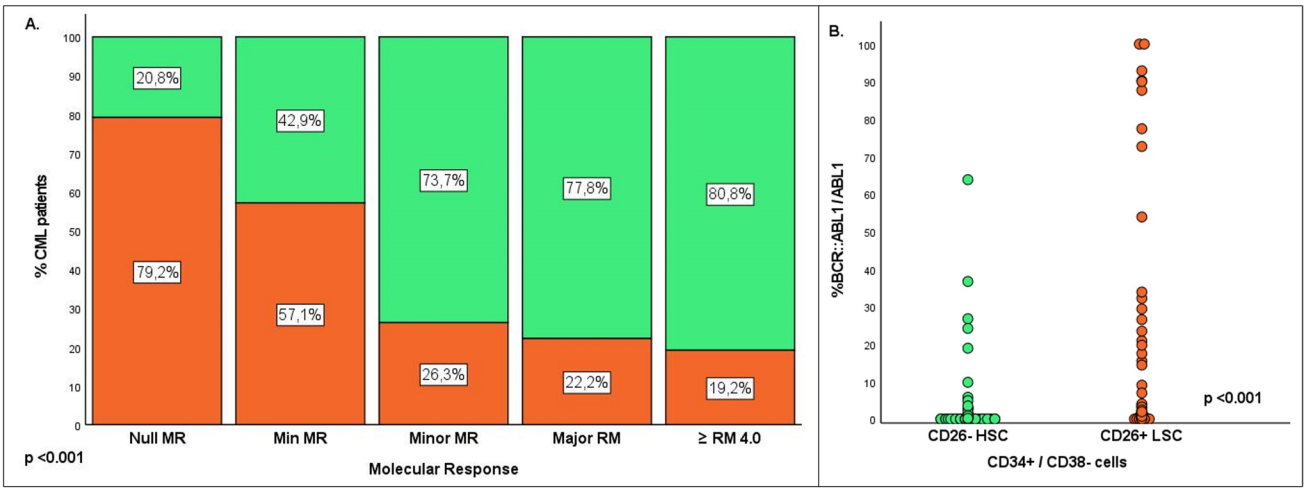

Fig. 2 Molecular Response and CD26 LSC in TKI treated CML patients. **A**. The X-axis represents the molecular response on the International Scale (IS), and the Y-axis represents the percentage of CML patients with presence (CD26 + LSC: CD45 + /CD34 + /CD38-/CD26 +) or absence (CD26-HSC: CD45 + /CD34 + /CD38-/CD26-) of LSC in each molecular category. The level of *BCR::ABL1* transcripts in IS was classified as a discrete variable in: ≥ 10% (Null MR), > 1% (Minimal MR), > 0.1% (Minor MR), ≤ 0.1% (Major MR) and ≤ 0.01% (including $MR^{4.0}$, $MR^{4.5}$ and $MR^{5.0}$). Chi Square Test p < 0.001. **B**. The X-axis represents CD26 + or CD26- and Y-axis the ratio *BCR::ABL1/ABL1* as a continuous variable. U Mann–Whitney test for non-parametric variables $p < 0.001$. The green color represents CD26- HSC and the orange CD26 + LSC. CML, chronic myeloid leukemia; HSC, hematopoietic stem cell; LSC, leukemia stem cell

the analysis revealed statistically significant differences ($p = 0.046$), the low correlation coefficient (r = 0.187) indicated a weak relationship between these variables, reflecting a poor predictive value (Fig. 3).

The analysis of the absolute number of CD26 + cells/µL over time, from diagnosis to 24 months of treatment, showed substantial reductions at 3 and 6 months (0.5 and 0.04 cells/µL, respectively) compared to diagnostic values (7.2 cells/µL). Follow-up at 9, 12 and ≥ 24 months showed minor fluctuations, suggesting variability in the counts of CD26 + LSC during treatment. However, these reductions were not statistically significant (KW, $p = 0.102$). Nevertheless, when analyzing the frequency of CD26 + LSC at different time points from diagnosis

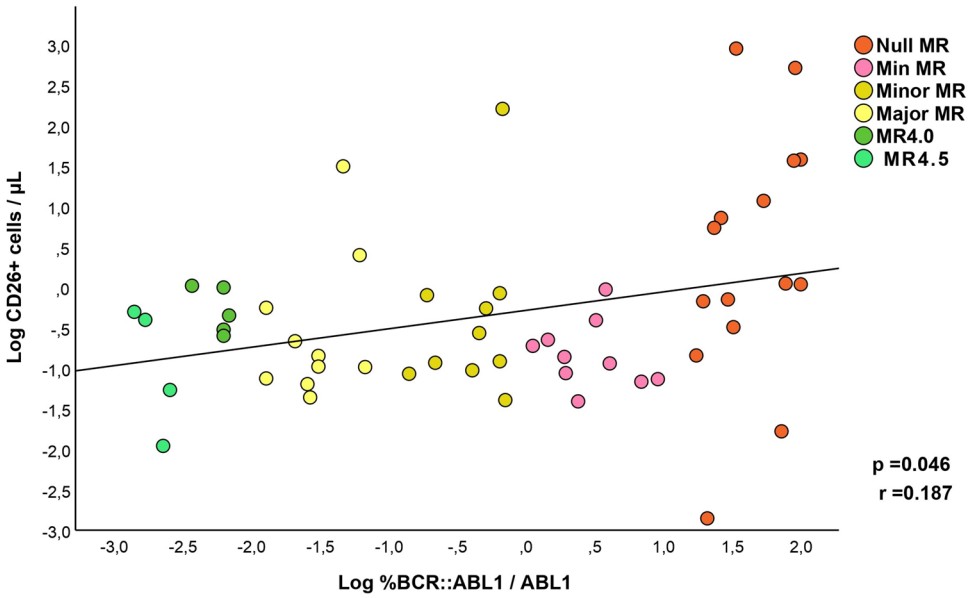

Fig. 3 Kendall rank correlation coefficient and scatter plot between the percentage of *BCR::ABL1* transcripts and CD26 + cells/µL. Both variables are transformed to the logarithmic scale for a better visualization of the data. Linear regression (black line). r: Kendall rank correlation coefficient. MR: Molecular response. ≥ $MR^{4.5}$ includes all cases with ≤ 0.0032 *BCR::ABL1*^{IS}

Table 3 CD26 + LSC frequency according to treatment time

Treatment time	CD34+/CD38- cells		CD26+
	CD26- *n* = 143	CD26+ *n* = 67	Md cells/µL (range)
Diagnosis (%)	1 (16.7)	5 (83.3)	7.20 (0.001–513.0)
3 months (%)	12 (46.2)	14 (53.8)	0.50 (0.039–890.0)
6 months (%)	11 (73.3)	4 (26.7)	0.04 (0.017–0.073)
9 months (%)	10 (62.5)	6 (37.5)	0.23 (0.041–160.0)
12 months (%)	8 (80.0)	2 (20.0)	0.13 (0.117–0.144)
≥ 24 months (%)	101 (73.7)	36 (26.3)	0.29 (0.011–31.18)
p value	$\chi^2 = 0.006$		KW = 0.102

Bold Values represent highly significant p-values (<0.001)

Md, Median; KW, Kruskal Wallis test; X^2, Chi square distribution

to 24 months, a significant decrease was observed (χ^2, $p < 0.001$) (Table 3).

CD26 + LSC dynamics and molecular response in TFR patients

Levels of CD26 + LSC and *BCR::ABL1* transcripts were analyzed on a small group of 16 patients from the Argentine Stop Trial (NCT05926128), which included only 12 females and 4 males, with a median age of 60.1 years (range: 49.5–85.0) at the time of treatment withdrawal. Prior to discontinuation, first-line TKI therapy consisted of 11 cases on imatinib, 2 on nilotinib, and 3 on dasatinib. All these cases were monitored during 2 years in TFR according to the AST protocol; after this period, they were analyzed in our laboratory, which means that only late relapses could be evaluated in this group. The study of LSC dynamics during follow-up revealed their reappearance in only 2 patients at different follow-up points (2.9 and 4.7 years in TFR). However, no LSC were observed in subsequent studies, suggesting that the fluctuations detected without loss of MMR do not imply loss of TFR. In this group, only 6% (1/16) of cases experienced molecular relapse after 4.2 years of follow-up (Table 4).

The 15 patients in TFR in the real-life settings comprised 6 females and 9 males, with a median age of 57.8 years (range: 24.3–87.6) at the time of treatment discontinuation. Twelve patients had received imatinib, 2 nilotinib, and 1 dasatinib prior to discontinuation. During follow-up, 3 cases showed molecular relapse between 2 to 5 months after discontinuation; however, only 1 case exhibited the presence of CD26 + LSC. The remaining 12 patients continue in TFR, and in 2 of these cases, LSC were observed without molecular relapse at 1.1 and 1.8 years into TFR (Table 4).

Discussion

CD26 + LSC are specific markers of CML, absent in healthy individuals and other myeloid neoplasms [8, 16]. Our data indicate a significant decrease in CD26 + LSC as patients achieved deeper molecular responses, consistent with previous findings [8, 16, 18, 25–27]. No significant differences were observed in their frequency among patients treated with different TKIs across molecular response categories, in line with previous data [18, 28, 29]. However, nilotinib seemed to induce a more pronounced decrease in CD26 + levels, suggesting a potentially enhanced treatment response.

CD26 + LSC expression is most frequently detected at diagnosis or in patients with poor treatment response

Table 4 CD26 + LSC in CML patients in treatment-free remission

Sex	TKI Stop Age (y)	Sokal score	TKI treatment (mg)	Treatment duration (y)	TFR[a] duration (y)	*BCR::ABL1* (%)	Molecular response[a]	[a]CD34+/CD38-/CD26+ (cells/μL)
Real-Life TFR patients (n = 15)								
F	69.7	Int 0.9	Ima (400)	7.4	0.2	UMR	$MR^{4.5}$	Absent
M	75.1	Low 0.7	Ima (400)	12.6	0.2	**0.164**	**Minor MR**	Absent
M	87.6	Int 1.0	Ima (400)	16.8	0.4	UMR	$MR^{4.5}$	Absent
M	45.7	Low 0.7	Ima (400)	6.8	0.4	**2.580**	**Min MR**	1.704
F	57.3	High 3.0	Ima (400)	12.2	0.5	**9.200**	**Min MR**	Absent
M	24.3	Int 1.0	Ima (400)	8.9	0.6	0.007	$MR^{4.0}$	Absent
M	64.3	High 1.4	Ima (400)	15.0	0.7	UMR	$MR^{4.5}$	Absent
F	81.8	Int 0.8	Nilo (600)	11.3	1.1	UMR	$MR^{4.0}$	0.15[b]
M	77.4	Int 0.8	Ima (400)	8.1	1.4	0.048	Major MR	Absent
M	63.8	Low 0.7	Ima (400)	9.5	1.6	UMR	$MR^{4.5}$	Absent
F	41.8	Int 0.8	Ima (400)	4.7	1.9	UMR	$MR^{4.5}$	Absent
F	35.6	Int 0.9	Ima (400)	14.4	2.1	UMR	$MR^{4.5}$	Absent
M	44.9	Int 0.8	Nilo (600)	9.3	2.1	0.024	Major MR	Absent
M	50.7	Int 1.0	Ima (400)	18.0	2.8	UMR	$MR^{4.5}$	Absent
F	42.6	High 1.5	Dasa (100)	5.3	3.1	UMR	$MR^{5.0}$	Absent[c]
	Md = 57.3			Md = 9.5	Md = 1.1			
Argentina Stop Trial patients (n = 16)								
F	65.4	Int 0.9	Ima (400)	16.1	2.2	UMR	$MR^{4.5}$	Absent
F	49.5	Low 0.6	Ima (400)	12.3	2.4	0.007	$MR^{4.0}$	Absent
F	52.6	Int 0.8	Ima (400)	15.3	2.5	UMR	$MR^{4.0}$	Absent
F	59.8	Int 1.0	Ima (400)	10.8	2.5	UMR	$MR^{4.0}$	Absent
M	66.0	Low 0.7	Nilo (600)	7.3	2.7	UMR	$MR^{4.0}$	Absent
M	49.8	Int 0.9	Ima (400)	11.8	2.9	0.004	$MR^{4.0}$	Absent
F	51.6	Int 0.9	Dasa (100)	11.8	2.9	UMR	$MR^{4.5}$	Absent
F	55.6	Int 0.8	Ima (400)	13.3	3.8	0.005	$MR^{4.0}$	Absent[c]
F	60.4	Low 0.7	Ima (400)	6.8	4.1	**0.170**	**Minor MR**	Absent
F	64.4	Low 0.7	Nilo (600)	16.4	4.3	UMR	$MR^{4.5}$	Absent
M	72.8	High 1.3	Ima (400)	12.8	4.5	UMR	$MR^{5.0}$	Absent
F	52.0	Int 0.8	Ima (400)	8.7	4.7	UMR	$MR^{4.0}$	Absent
F	85.0	High 1.2	Ima (400)	13.2	4.7	UMR	$MR^{5.0}$	0.05[b]
F	59.1	Int 1.0	Dasa (100)	10.5	4.7	UMR	$MR^{4.5}$	Absent
F	67.1	Int 0.8	Dasa (100)	11.5	4.9	UMR	$MR^{5.0}$	Absent
M	63.8	Int 0.8	Ima (400)	11.4	6.3	UMR	$MR^{4.5}$	Absent
	Md = 64.2			Md = 11.8	Md = 4.0			

y, years; Md, median; Int, Intermediate; Ima, Imatinib; Nilo, Nilotinib; Dasa, Dasatinib; MR, Molecular Response; Min, Minimal; UMR, Undetectable MR

[a]The analysed data correspond to the last sample evaluated. Patients who experienced loss of TFR are shown in bold

[b]Reappearance of LSC was observed without loss of molecular response

[c]In previous studies, LSC was observed without loss of molecular response

[18, 26, 30]. In our cohort, 20% of patients with null MR had undetectable CD26 + LSC levels, indicating that high *BCR::ABL1* transcript levels (> 10% $BCR::ABL1^{IS}$) do not always correlate with detectable CD26 + LSC. This could reflect the independence of CD26 + LSC from *BCR::ABL1* expression [31] and the high cellular heterogeneity at diagnosis or in null MR cases. Culen et al. [32] reported significant variability in the proportions of CD26 + LSC and CD26- HSC in BM samples at diagnosis, categorizing patients from high to absent CD26 + LSC groups. For those with minimal LSC levels, only highly sensitive RT-qPCR was able to differentiate neoplastic from normal cells. Similarly, Warfvinge et al. [33] combined MFC with single-cell analysis to explore heterogeneity within the LSC population

at CML diagnosis. This approach identified subpopulations with myeloid and proliferative characteristics that responded favorably to TKI therapy, in contrast to primitive and quiescent LSC, which exhibited TKI resistance.

We observed higher proportions of CD26 + LSC in patients with elevated *BCR::ABL1* transcript levels. Previous studies have shown no correlation between the absolute number of LSC/μL and *BCR::ABL1* levels [18, 27–29, 34]. However, we noted a weak association (r = 0.187) among these variables, consistent with previous findings [25]. In our cohort, CD26 + LSC/μL showed a substantial reduction at 3 and 6 months of therapy, with minor fluctuations observed between 9 and 24 months. Bocchia et al. [28] reported similar findings in a multicenter study (Prospective Flowers), noticing a significant drop in CD26 + LSC/uL levels at 3 months and minimal fluctuations thereafter. In DMR cases, LSC presence showed no correlation with *BCR::ABL1* levels, suggesting that MFC may detect LSC in either proliferative or quiescent states, regardless of *BCR::ABL1* transcription levels. Furthermore, the absence of LSC did not correlate with *BCR::ABL1* ratio, indicating that molecular studies are more informative about treatment response, especially in DMR. Our data, in line with other publications, suggest that while DMR patients exhibit very low number of CD26 + LSC in PB, these cells can persist in the BM. Ilhan et al. [26] demonstrated that DMR patients on TKI treatment had both undetectable *BCR::ABL1* transcripts and CD26 + LSC/μL in PB, but quiescent LSC persisted in BM, potentially leading to treatment failure and relapse after discontinuation.

Approximately half of the patients in TFR lose MMR and must resume treatment. Since quiescent CD26 + LSC are the reservoir of CML, assessing their presence can serve as prognostic marker in TKI discontinuation. We performed MFC and qPCR assessments on two different TFR patient groups. In the AST protocol group [21, 22], our data indicate fluctuations in the LSC in 2 cases without molecular recurrence and only one patient (1/16) showed loss of TFR after 4 years. Rousselot et al. [35] reported that late molecular recurrences in patients with over 2 years of TFR account for approximately 14% of all molecular relapses. The study of real-life TFR cases showed molecular relapse in 3 out of 15 cases, with only one of them exhibiting LSC presence. Although the cohort of TFR patients studied was small, the lack of correlation between molecular relapse and LSC recurrence suggests that LSC presence does not predict failure after treatment discontinuation. Pacelli et al. [36] found that most patients in stable TFR exhibited CD26 + LSC in PB with significant variability; however, there was no correlation with relapse incidence or a predictive threshold for LSC levels related to TFR loss. Furthermore, TFR loss can occur even when LSC are undetectable, suggesting that other factors, such as the host immune system, are crucial for maintaining it. Some studies suggest that high levels of NK cells may eradicate LSC and enhance adaptive immune responses [37–40].

In conclusion, our work shows that LSC are frequently observed in patients with poor molecular response, with no differences between TKI treatments. Consequently, as patients achieve DMR, CD26 + LSC presence significantly decreases. However, the study of CD26 + LSC in TFR showed a lack of predictive value, as LSC recurrence during follow-up did not correlate with molecular relapse. Although CD26 + LSC are the reservoir of CML, their persistence or reappearance may not be a predictor of prognosis or relapse after treatment discontinuation, questioning their clinical relevance in TFR.

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Author contributions MFC performed the molecular research, the statistical analysis and interpretation of results. MP processed all the samples by Flow cytometry and collaborated with the illustrations. MJT participated in the analysis of data and contributed with the writing of the article. BM, AE, RM, FN, CP, VV, MJF, IE contributed with clinical data of patients and their follow-up according to current guidelines. RB and IB developed the study, conceived and planned the experiments and wrote the article. All authors critically revised the manuscript and approved its final version.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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