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# Tracking leukemic residuals: dissecting the inverse relationship between CD26+ stem cells and extracellular *BCR::ABL1* transcript in Chronic Myeloid Leukemia (CML)

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## Abstract

Chronic myeloid leukemia (CML) persists due to leukemic stem cells, notably the CD26+ subset. We investigated correlations between circulating CD26+ leukemic stem cells (LSCs) and *BCR::ABL1* transcripts in an extracellular vesicle-enriched secretome (EVES) from plasma samples of 44 CML patients. EVES were characterized and *BCR::ABL1* quantified via digital PCR. We observed an inverse correlation between CD26+LSC counts and EVES *BCR::ABL1* levels, especially in deep molecular responders (DMR). CD26+LSCs were elevated in patients in treatment-free remission (TFR), while EVES *BCR::ABL1* levels were higher in those receiving therapy. These findings suggest distinct dynamics between LSC populations and vesicle-mediated transcript release, with potential implications for CML monitoring and prognosis.

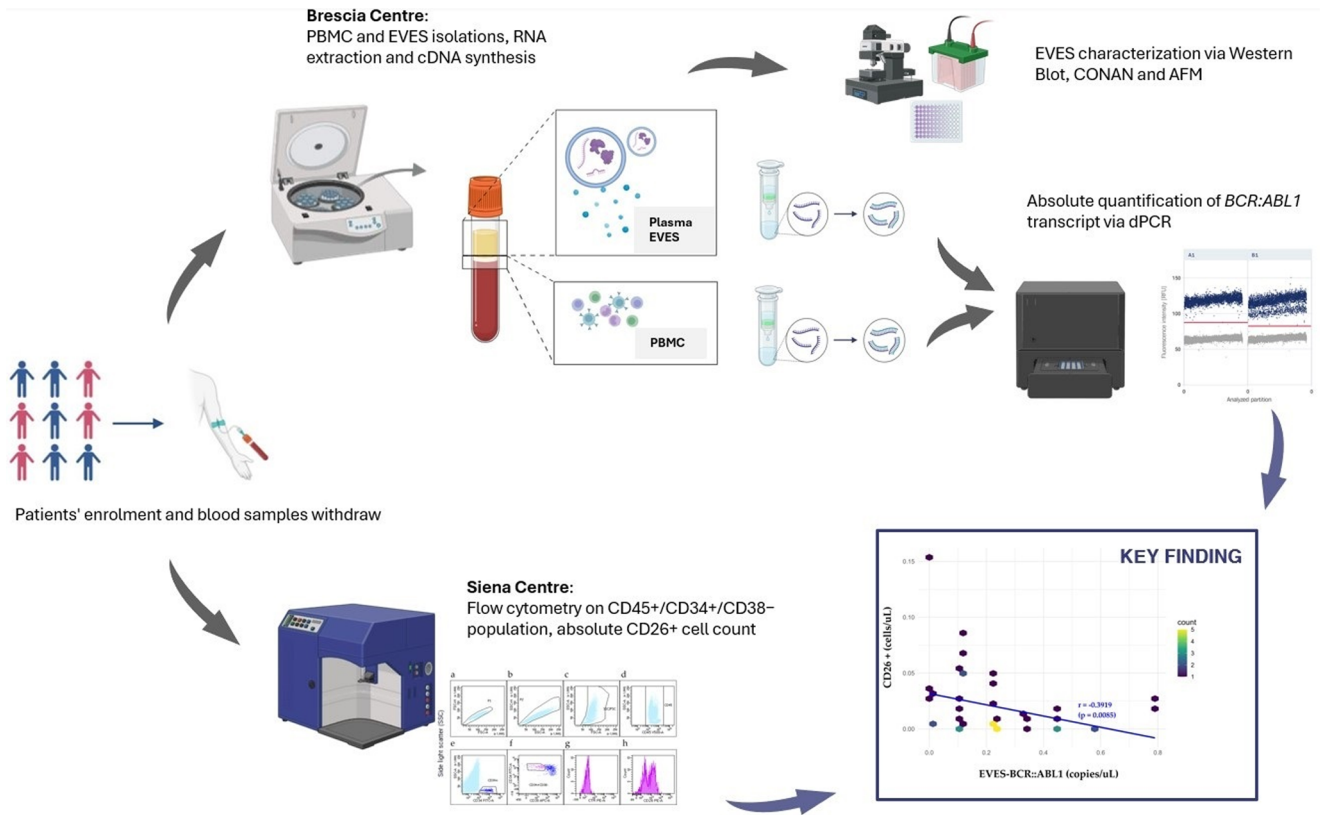
**Key words:** chronic myeloid leukemia; leukemic stem cells; CD26; EVES; *BCR::ABL1*.

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## Graphical abstract



## Significance Statement

This study reveals a novel inverse relationship between circulating leukemic stem cells and *BCR::ABL1* transcript carried by extracellular vesicles in chronic myeloid leukemia. The findings suggest that different leukemic cell populations may be active depending on treatment status, with vesicle-associated transcript potentially reflecting disease activity even when traditional cell-based markers are reduced. These insights highlight the complex dynamics between stem cells and extracellular communication in leukemia and point to the potential of vesicle-derived biomarkers as tools for disease monitoring, risk assessment, and therapy guidance.

## Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the presence of the *BCR::ABL1* fusion gene.<sup>1</sup> Despite the success of tyrosine kinase inhibitors (TKIs) in managing the disease, leukemic stem cells (LSCs) persist and contribute to relapse. A subset of LSCs, found in peripheral blood and bone marrow at comparable counts, can be identified via flow cytometry by the CD26 membrane marker, as proved in previous observations,<sup>2-4</sup> while CD93 identifies a subpopulation of quiescent stem cells.<sup>5</sup> Additionally, circulating CD26+ cells are Ki67+, indicating active proliferation. Despite their intriguing potential significance, the CD26+LSC number has not yet been clearly correlated with other biological or clinical parameters.<sup>6-9</sup> In addition to cellular biomarkers, small extracellular vesicles (EVs) have emerged as a valuable tool for *BCR::ABL1* quantification and disease monitoring.<sup>10</sup> These vesicles carry *BCR::ABL1* transcripts, enabling a minimally invasive approach to detect active leukemic residual cells. Moreover, the stability of EVs' cargo, protected by the lipid membrane, supports its use as a reliable biomarker for tracking

disease burden, predicting relapse, and assessing treatment response.<sup>11-15</sup> Based on these insights, we investigated for the first time the presence of a correlation between *BCR::ABL1* transcript levels contained in an extracellular vesicle-enriched secretome (EVES) fraction and the amount of circulating CD26+ cells in a preliminary cohort of CML patients. EVES fraction was selected because it is enriched in EVs, allowing for faster and more efficient analysis of patient samples.

## Methods

## Patients' characteristics

A total of 44 adult patients affected by CML who achieved at least major molecular response (MMR) under TKIs provided their written informed consent and were enrolled in the study. General characteristics of the entire cohort are reported in Table 1. Among them, 10/44 (22%) were in MMR and 34/44 (78%) were in deep molecular response (DMR) following International Scale (IS) assessed by RT-qPCR. All patients were analyzed once by sampling 15 mL of peripheral blood (PB).

**Table 1.** Characteristics of the studied population.

Generic variables	
Sex (no. M–no. F)	29–15 (66%–34%)
Age at diagnosis (median (range))	52 y (19 y–90 y)
Age at sampling (median (range))	67 y (30 y–91 y)
MR	
MR3.0	10/44 (23%)
MR4.0	10/44 (23%)
MR4.5	4/44 (9%)
MR5.0	20/44 (45%)
Therapy	
First generation TKI	19/44 (43%)
In first-line therapy	2/19 (10%)
Second generation TKI	13/44 (30%)
In first-line therapy	2/13 (15%)
TFR	12/44 (27%)
Therapy duration in months (median (range))	
First generation TKI	62 (16–285)
Second generation TKI	47 (7–119)
TFR	64 (1–210)

Abbreviations: F= female, M= male, MR= molecular response, TFR = treatment-free remission, TKI = tyrosine kinase inhibitor, y= years.

### EVES characterization

EVES characterization included Western blot (WB) analysis, Colloidal NANoplasmonic assay (CONAN), and Atomic Force Microscopy (AFM). Total protein concentrations of whole plasma and EVES were evaluated by Bradford assay (Bio-Rad) following the manufacturer's instructions.

For WB, mouse anti-Flotillin 1 (FLOT-1) and mouse anti-CD63 were used as EV-specific markers and anti-apolipoprotein 1 (Apo A1) for high-density lipoproteins (HDL) was detected.

The EVES preparations were checked for purity from soluble protein contaminants (SPC) using the CONAN assay, a recognized method to determine the purity of EV preparations that is based on the clustering of gold NPs onto lipid membranes.<sup>16,17</sup> The CONAN assay was performed as previously described by Zendrini et al.<sup>18</sup>

To study EVES morphology, AFM imaging was performed using a Nanosurf NaioAFM equipped with Multi75AI-G probes (Budget sensors).<sup>19</sup> Detailed procedures are described in [Supplementary Materials](#).

### Flow cytometry analyses

A volume of 5 mL of EDTA PB samples were centralized via express courier to the Flow Cytometry lab in Siena and analyzed within 24 h. Circulating CD26+ cells were evaluated via standardized multiparametric flow cytometry analysis of the CD45+/CD34+/CD38– population using a four-color staining protocol. The median absolute number of CD26+ cells/μL was calculated as follows: (no. WBCs/μL) × (% of CD34+/CD38–/CD26+ within CD45+ cells); 2 × 10<sup>6</sup> leukocytes were incubated with a custom-made, lyophilized, pre-titrated antibody mixture in a test tube (BD™ Lyotubes, BD) including CD45 V500 (BD Pharmingen clone 2D1), CD34 FITC (BD Pharmingen clone

581), CD38 APC (BD Pharmingen clone HIT2), and CD26 PE (BD Pharmingen clone M-A261) antibodies, as well as a BD stain control tube lacking the CD26. Samples were analyzed using a FACSLytic flow cytometer and FACSuite software (BD Biosciences). The gating strategy is shown in [Supplementary Materials](#).

### EVES separation and dPCR analysis

A volume of 10 mL of EDTA PB samples were centralized in Brescia Centre and centrifuged at 1560 g for 15' to separate the plasma fraction. EVES was obtained from plasma samples using Plasma/serum Exosome Purification and RNA isolation Mini kit (#58300, Norgen Biotek Corporation) starting from 1.5 mL of plasma, following the manufacturer's instructions. EVES was eluted in 200 μL of ExoR buffer, 100 μL were used for RNA extraction, while the remaining 100 μL were used for its characterization. Total EVES RNA was eluted in 50 μL. Finally, cDNA was synthesized via RNAUscript Reverse Transcriptase kit (LeGene Bioscience) according to the manufacturer's instructions with final reaction volume of 20 μL. EVES *BCR::ABL1* (Hs03024541\_ft, FAM-MGB) transcript was quantified via digital PCR (dPCR) using the QuantStudio Absolute Q Digital PCR System (Life Technologies). Briefly, 5 μL of EVES cDNA were loaded on a dPCR plate together with 1.8 μL of Absolute Q™ DNA dPCR Master Mix (5X), 0.45 μL of 20X TaqMan-MGB-FAM probe assay and 1.75 μL of nuclease-free water (Qiagen). Thermocycling conditions included a denaturation at 95 °C for 8 minutes, followed by 40 cycles of 90 °C for 15" and 60 °C for 1', and a final extension step at 60 °C for 2'. dPCR plates were automatically analyzed via Applied Biosystems™ QuantStudio™ Absolute Q Digital PCR Software, version 6.3.2.

*BCR::ABL1* transcript was quantified by dPCR also on PB cells analyzing 50 ng of cDNA, as previously described.<sup>20</sup>

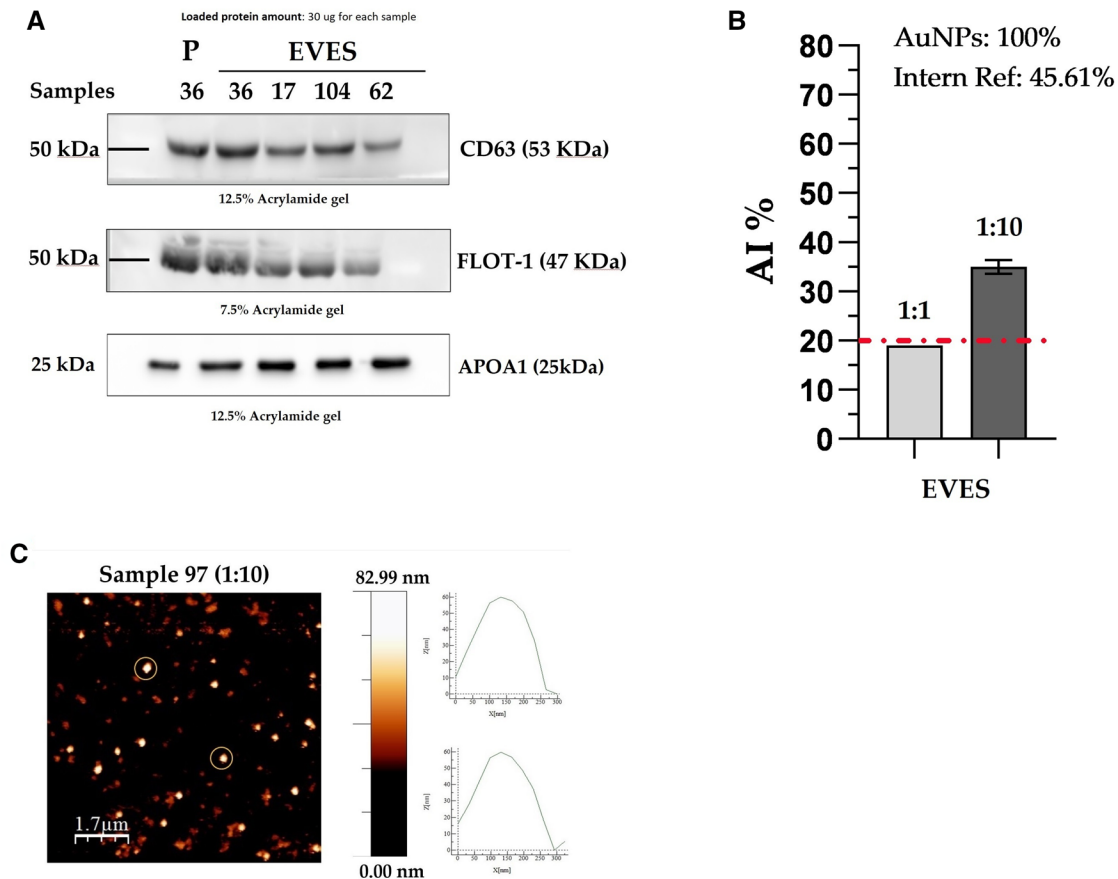
### Statistical analysis

The Spearman correlation coefficient was used to explore potential relationships among the measurable variables, with a 95% confidence interval (CI). Linear correlations were evaluated using RStudio (version 4.5.1). No adjustments for multiple comparisons were applied to either the Spearman or linear correlation analyses, as they were exploratory in nature. Additional statistical analyses were performed using two-tailed, unpaired *t*-tests in GraphPad Prism (version 8.4.3).

## Results

### EVES characterization via WB, CONAN, and AFM

The characterization overall results confirmed the enrichment of EVs (CD63 and FLOT-1 presence) together with other extracellular nanoparticle such as HDLs in the preparations (Apo A1 presence). To ensure the presence of EVs in the starting material, the markers were further tested on whole plasma ([Figure 1A](#)). CONAN results indicate that samples present an AI% lower than 20% at the minimal dilution of the sample (1:1) and remained below the 20% (dotted line) even at higher dilutions, but with increasing AI% ([Figure 1B](#)). The EVES size and morphology were determined by AFM. In the EVES isolated from the characterized samples, we found spherical objects with diameters ranging from tens to hundreds of nanometers, matching the typical size of extracellular vesicles ([Figure 1C](#)). The overall



**Figure 1.** Characterization of extracellular vesicle-enriched secretoma (EVES) obtained from plasma samples. (A) Biochemical characterization via western blot. EVES samples (30 μg) and whole plasma (P) (30 μg) were analyzed for the presence of CD63 and FLOT-1, extracellular vesicles (EV) markers, and Apo A1 as high density lipoproteins (HDL) marker. (B) Determination of the presence of soluble protein contaminants (SPC) with the CONAN assay. Aggregation Index ratios (AI%) of serial dilution (1:1, 1:10) of a representative sample are shown in the graph. (C) Representative atomic force microscopy (AFM) images illustrating the morphology and profile of EVES nanoparticles in the preparations derived from one plasma sample (dilution 1:10).

results confirmed the enrichment of EVs together with other extracellular nanoparticle such as HDLs in the preparations.<sup>21</sup> Samples contained a negligible amount of SPC and thus can be considered depleted of protein contaminants (Figure 1).

### CD26+LSC and BCR::ABL1+ EVES analysis

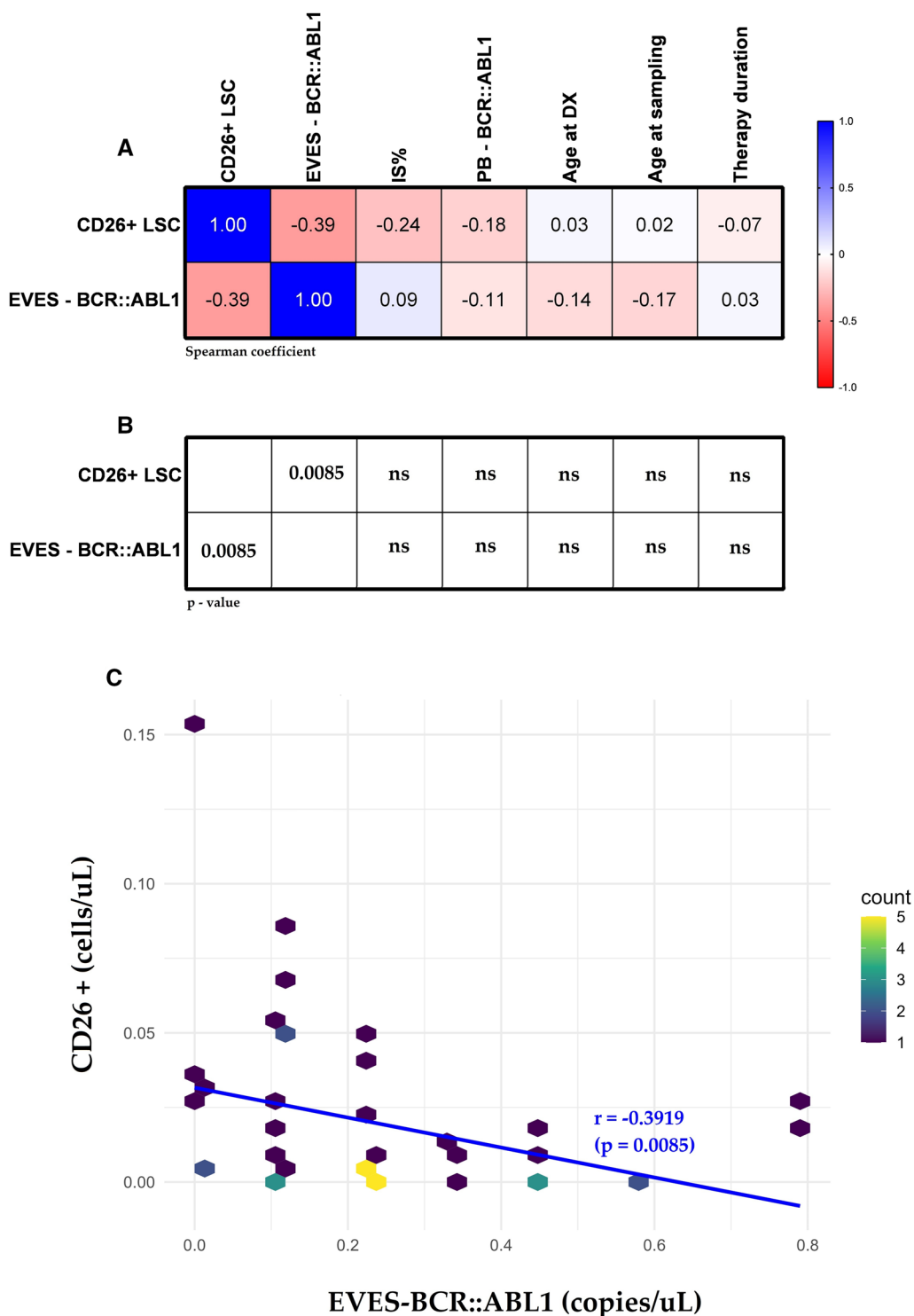
In our cohort, the median number of circulating CD26+LSC was 0.00625 cells/μL (range: 0–0.1565). A total of 14 patients (32%) presented undetectable levels of circulating CD26+LSC. On the other hand, the median absolute quantification of EVES BCR::ABL1 transcript was 0.230 copies/μL (range: 0–0.790). In this case, a total of five patients (14%) presented undetectable levels of EVES BCR::ABL1 transcript.

Consistent with previous studies, no significant correlation was observed between the number of circulating CD26+LSC or EVES BCR::ABL1 transcript and BCR::ABL1 levels expressed as IS% or the molecular response (MR) class assessed on PB cells via RT-qPCR. Similarly, the amount of BCR::ABL1 assessed in PB cells using dPCR did not correlate with the number of circulating CD26+LSC nor with EVES-BCR::ABL1 levels. Finally, correlations between the number of circulating CD26+LSC or EVES BCR::ABL1 transcript and age at diagnosis, age at sampling, and therapy duration were also evaluated and resulted not statistically significant, as shown in Figure 2A and B.

Conversely, our findings indicate an inverse correlation between the number of circulating CD26+LSC and the level of EVES BCR::ABL1 transcript, as reflected by the correlation coefficient  $r = -0.3919$  ( $P = 0.0085$ ) (Figure 2C). Furthermore, the significance improves when considering only patients in DMR ( $r = -0.4478$ ,  $P = 0.0079$ ).

Additional correlations with potential confounding factors, such as prior lines of TKI therapy and cytogenetic risk, could not be assessed in this cohort. None of the patients presented with additional chromosomal abnormalities (ACA) beyond  $t(9; 22)$ . Among those in TFR (12/44), all had received only a single line of TKI prior to discontinuation. Similarly, 88% (28/32) of patients who remained on TKI therapy were still receiving first-line treatment.

In addition, we evaluated whether the number of circulating CD26+LSC or EVES BCR::ABL1 transcript are influenced by therapy. Among patients with at least one detectable circulating CD26+LSC (30/44; 68%), a statistically significant increase in leukemic stem cell count was observed in those in TFR compared to patients receiving TKI therapy ( $P = 0.0123$ ; Figure 3A). The opposite trend, despite not statistically significant, was observed in the sub-cohort of patients with at least one detectable molecule of EVES BCR::ABL1 transcript (38/44, 86%) (Figure 3B).

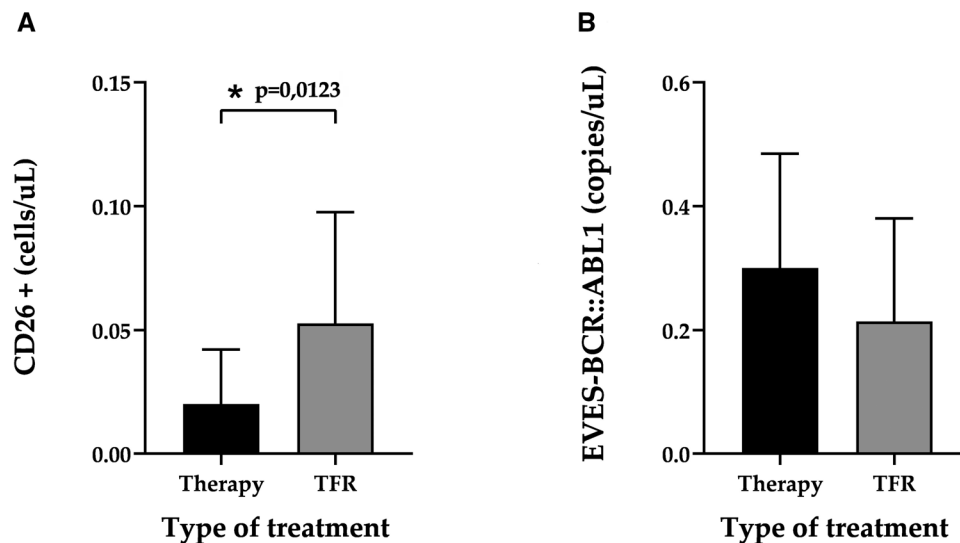


**Figure 2.** Nonparametric Spearman correlation matrix (95% CI) assessing associations between CD26+ leukemic stem cells (LSCs), EVES-BCR::ABL1, IS%, PB-BCR::ABL1, age at diagnosis, age at sampling, and therapy duration. (A) Spearman correlation coefficients, with color intensity ranging from red (negative correlation) to blue (positive correlation). (B) Corresponding P-values for each correlation. (C) Linear relationship between the two analyzed variables, with data points colored according to local density. The density plot highlights regions with higher sample concentration, allowing visualization of trends, clusters, and potential outliers in the data distribution (IS = International Scale, DX = diagnosis, PB = peripheral blood, ns = not significant).

### Discussion and conclusion

As previously demonstrated, CD26+ cells are present in comparable numbers in peripheral and bone marrow blood, and these proliferating Ki67+ LSCs significantly decline in response to TKIs treatment<sup>3,22</sup> and may serve as prognostic tools.<sup>23-25</sup>

Despite that, their significance, specific biological role, and clinical impact are still matter of debate. In fact, no specific correlation has been yet reported between the number of circulating LSC and other biochemical parameters in CML. For the first time, our results show an inverse relationship between the



**Figure 3.** Correlation of the number of circulating CD26+ leukemic stem cells (LSCs) (A) and the number of EVES *BCR::ABL1* transcript (B) in patients stratified by treatment status, treatment-free remission (TFR) versus ongoing tyrosine kinase inhibitors (TKI) therapy.

number of circulating CD26+LSCs and the amount of EVES *BCR::ABL1* transcript, suggesting that EVES *BCR::ABL1* levels increase as CD26+ cell counts decline. This could indicate, hypothetically, that a distinct leukemic population lacking CD26 expression may emerge and become functionally dominant as the CD26+LSCs decrease, actively releasing extracellular nanoparticles loaded with *BCR::ABL1* transcript. Alternatively, the declining CD26+ cells may themselves initiate a compensatory mechanism that enhances the release of *BCR::ABL1*-loaded extracellular vesicles, possibly as a response to cellular stress, therapeutic pressure, or changes in the leukemic microenvironment. Additionally, the amount of circulating CD26+LSCs appears to be influenced by TKI therapy, indicating a potential interplay between treatment status and leukemic cell behavior. Other groups have demonstrated that both patients undergoing TKI therapy and those in TFR harbor residual CD26+LSCs. However, quantification of these cells does not appear to correlate with the risk of relapse.<sup>3,6</sup> Moreover, these circulating CD26+ cells are actively proliferating and may remain sensitive to TKI therapy. Therefore, it is plausible that in the absence of treatment, their proliferation is favored. The correlation between CD26+LSCs and vesicular *BCR::ABL1* transcripts requires further investigation, particularly to determine the origin of these *BCR::ABL1* transcript-loaded vesicles. In fact, the inverse correlation suggests that the vesicles could derive from circulating precursors that need to be characterized. These findings are preliminary and need to be validated in larger, independent patient cohorts. Future studies should include independent cohorts, larger sample sizes, longitudinal data from patients, and mechanistic assays to confirm the biological relevance of these observations. Moreover, our cross-sectional study design does not allow us to draw conclusions regarding causality or the predictive value of EV-associated *BCR::ABL1* transcripts in CD26+LSCs. Potential confounding factors, including prior lines of TKI therapy and cytogenetic risk categories, could influence both CD26+LSCs counts and EV-associated *BCR::ABL1* transcript levels and should be considered in a

larger cohort. Finally, a thorough comprehension of the prognostic implications of this data needs to be clarified because it may provide important information about the biological basis of CML and the course of the disease.

### Supplementary material

Supplementary material is available at *Stem Cells Translational Medicine* online.

### Funding

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### Conflicts of interest

None declared.

### Data availability

All data are available by corresponding author upon request.

### Ethics approval and patient consent statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committees of Siena and Brescia (Italy). Protocol code “AIRC IG 20133” approved on 19/02/2018 in Siena and “NP-DPCR-CML\_1603” approved on 10/06/2016 in Brescia. Written informed consent was obtained from all subjects involved in the study.

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N/A.

## Clinical trial registration

The present study was a non-pharmacological trial approved by local IRBs and the registration on public registries was not required.

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