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Chronic myeloid leukemia: from targeted therapy to treatment-free remission

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Chronic myeloid leukemia: from targeted therapy to treatment-free remission

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Modelling of immune response in chronic myeloid leukemia patients suggests potential for treatment reduction prior to cessation

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Introduction: Discontinuation of tyrosine kinase inhibitor (TKI) treatment is emerging as the main therapy goal for Chronic Myeloid Leukemia (CML) patients. The DESTINY trial showed that TKI dose reduction prior to cessation can lead to an increased number of patients achieving sustained treatment free remission (TFR). However, there has been no systematic investigation to evaluate how dose reduction regimens can further improve the success of TKI stop trials.

Methods: Here, we apply an established mathematical model of CML therapy to investigate different TKI dose reduction schemes prior to therapy cessation and evaluate them with respect to the total amount of drug used and the expected TFR success.

Results: Our systematic analysis confirms clinical findings that the overall time of TKI treatment is a major determinant of TFR success, while highlighting that lower dose TKI treatment for the same duration is equally sufficient for many patients. Our results further suggest that a stepwise dose reduction prior to TKI cessation can increase the success rate of TFR, while substantially reducing the amount of administered TKI.

Discussion: Our findings illustrate the potential of dose reduction schemes prior to treatment cessation and suggest corresponding and clinically testable strategies that are applicable to many CML patients.

KEYWORDS

chronic myeloid leukaemia (CML), treatment free remission (TFR), dose reduction, tyrosine kinase inhibitor (TKI), mathematical modelling

Introduction

Chronic myeloid leukemia (CML) is a malignancy of the hematopoietic stem cell. The introduction of tyrosine kinase inhibitors (TKI) revolutionized CML treatment as they target the causative BCR-ABL1 oncoprotein (1). Most patients respond well to TKI treatment and achieve sustained molecular remission, defined as low levels of *BCR-ABL1* mRNA (2, 3). Optimally responding patients treated with the first generation TKI imatinib achieve a 3-log reduction in *BCR-ABL1* levels (denoted as major molecular remission, MR3) after a median of 18 months and a 4-log reduction (MR4) after a median of 45 months (4). Related to this molecular response, the overall life expectancy of CML patients approaches that of a healthy reference cohort (5). However, continuous treatment with TKI can impose adverse effects and is costly (6). Therefore, several studies have investigated whether optimally responding patients can stop TKI therapy yet remain in treatment free remission (TFR) (7, 8). Consistently, about 50% of patients can achieve sustained TFR while 50% develop molecular disease recurrence, typically within two years of stopping (8–11). Although longer treatment duration and deep molecular remission prior to stopping are favorable prognostic markers of TFR (8), it is still not possible to prospectively identify patients likely to undergo disease recurrence and thus exclude them from TFR attempts.

The reasons why some patients remain in TFR while others present with molecular recurrence are not clear. Complete eradication of CML cells during TKI therapy is unlikely, as this occurs over much longer time scales (12, 13), if at all (14). Moreover, some patients in sustained TFR still have measurable residual *BCR-ABL1* levels, suggesting that other factors sustainably control the remaining leukemic cells (15). There is accumulating evidence that the immune system contributes to this control (16–20), and there is evidence that TKI-driven reduction of leukemic cells shifts this balance and reactivates immune surveillance in CML (21, 22). This effect is further modulated once TKI treatment stops and might well influence the success of TFR (16, 23).

The integration of treatment data with underlying mathematical models can address the structural conditions necessary for a stable balance between leukemia remission and immunological control. We and others have shown that a bidirectional interaction between leukemia growth and anti-leukemic effects, such as a specific immune response, is a prerequisite to obtain stable remission scenarios (24–26). It remains to be investigated to what extent an adapted TKI treatment before stopping can maintain molecular remission while sufficiently stimulating the immune system to establish long term immune surveillance.

In this context, the DESTINY trial is of particular interest, as it specifically alters the TKI treatment schedule before patients stop TKI (27). Its study protocol includes a 12 month TKI reduction period to 50% of the original dose prior to complete cessation, which improved TFR rates to > 60%. We previously demonstrated that the initial dose reduction period is indeed informative to identify a subset of patients with a high risk for TFR failure (28), by showing that 87.9% of patients with highly increasing *BCR-ABL1* values during this time experienced a molecular recurrence, compared with only 27.5% recurrence in the group with no or low increase of *BCR-ABL1*. From this we concluded that rapidly increasing *BCR-ABL1* values during dose reduction strongly suggest TFR failure after TKI stopping.

The results of the DESTINY trial also raise the question whether other dose reduction strategies prior to stopping could further increase the overall success rate of TFR. To address this, we have adapted our previously suggested mathematical model of CML treatment and immune response (29) to describe extended CML time course data from the DESTINY trial. Thereby we obtain an indirect estimate of the leukemia-immune interaction of a larger patient cohort, which we further use to illustrate a novel modeling strategy allowing us to explore whether amended schedules of TKI application, including different schedules of dose reduction, can influence the overall success rate of TFR.

Materials and methods

Patient data

Our mathematical modelling approach is based on patient data from the DESTINY trial (NCT 01804985), whose primary results have been previously reported (27). The trial studied the effect of TKI reduction to 50% of the standard dose for 12 months prior to TKI cessation. All patients were previously treated with TKI monotherapy (either with imatinib, dasatinib or nilotinib) for at least three years and achieved stable molecular remission (MR3 in 49 patients and MR4 in 125 patients) for at least 12 months prior to entering the trial (see Supplementary Text S1, Supplementary Figure S1A). For the numerical analysis we use a logarithmic transformation of the *BCR-ABL1/ABL1* ratios (LRATIO = log10(*BCR-ABL1/ABL1*)).

In order to adapt the mathematical model to informative patient time courses, we applied several selection criteria to ensure a sufficient dose reduction step and enough measurements during early TKI treatment, dose reduction and follow up (Figure 1A). This yields a total of 67 time courses for a detailed analysis (denoted as *clinical reference data set*). Initial statistical assessment revealed no or minor differences between the original patient cohort and the reference data set with respect to initial LRATIO, treatment duration, follow-up duration, recurrence times and type of TKI (Supplementary Figure S1B-G). The selection process moderately increases the proportion of

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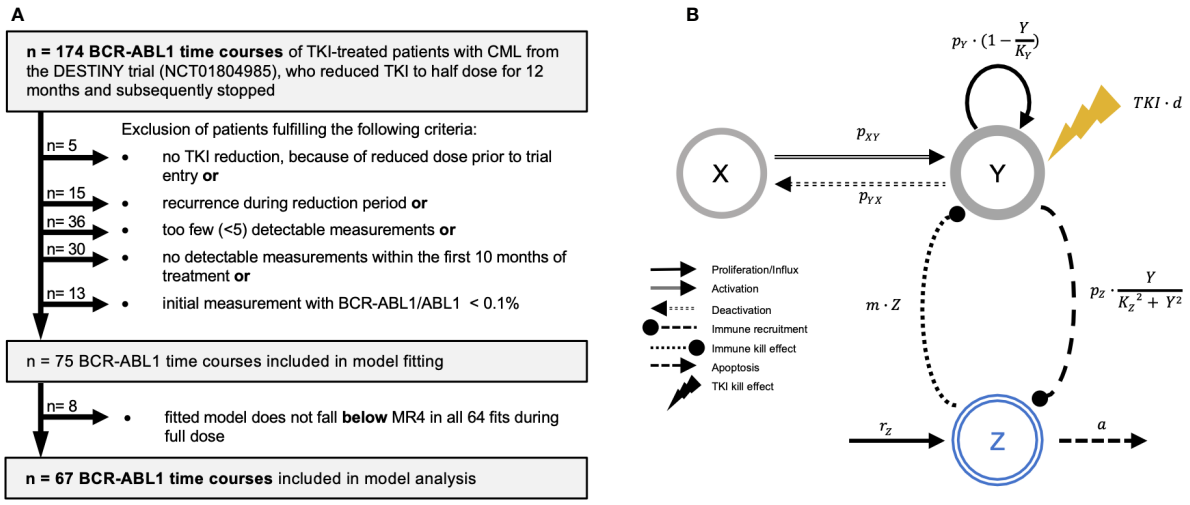


FIGURE 1
(A) Flow diagram indicating the strategy for patient selection. Patients were excluded when they did not undergo TKI reduction, as they entered the trial with a reduced dose already. Additionally, we excluded patients with less than 5 detectable BCR-ABL1 measurements, no detectable measurements, or an initial BCR-ABL1/ABL1-ratio below 0.1%. Furthermore, we excluded patients that presented with a recurrence during reduction period. After model fitting, we identified and excluded 8 patients, for which not all of the 64 simulation fits did predict BCR-ABL1 levels below MR4 during the full dose treatment period. **(B)** General scheme of the ODE model setup indicating the relevant cell populations and their mutual interactions (arrows with rate constants) that govern their dynamical responses. Leukemic cells can reversibly switch between the quiescent (*X*) and proliferating (*Y*) state with corresponding transition rates p_{XY} and p_{YX}. Proliferating cells divide with rate $p_Y \cdot (1 - \frac{Y}{K_Y})$. The TKI treatment has a cytotoxic effect *TKI* on proliferating cells (yellow lightning symbol, linearly depending on the dose *d*) while quiescent cells are not affected. Immune cells in *Z* have a cytotoxic effect (with rate *m*) on proliferating leukemic cells in *Y*. The proliferation of immune cells is stimulated in the presence of proliferating leukemic cells by an immune recruitment rate $p_Z \cdot \frac{Y}{K_Z^2 + Y^2}$. This nonlinear term describes an immune window, where the immune response is suppressed for high leukemic cell levels above the constant K_Z. Moreover, immune cells are generated by a constant production r_z and undergo apoptosis with rate *a* (see Materials and Methods).

CML recurrences from 38.5% in the original to 52.2% in the selected cohort. However, we are not primarily aiming to best mimic the DESTINY cohort but rather to identify patients for which we can obtain good and reproducible model fits.

Mathematical model of TKI-treated CML

We describe individual disease dynamics of CML patients (i.e. time course of *BCR-ABL1/ABL1* ratios) in terms of a mathematical model which comprises interactions between immune effector cells (*Z*), quiescent (*X*) and proliferating (*Y*) leukemic stem cells (Figure 1B). Herein, we extend the previously introduced model (29) based on ordinary differential equations (ODEs), to account for the intermediate dose reduction period. Assuming a linear dose-response relationship for the TKI treatment (30), the dosage factor *d* declines from *d=1* (full dose) to *d=0.5* or *0.25* (reduced dose) to *d=0* (therapy cessation). Details of the model are provided in Supplementary Text S2.

For comparison of the model simulation results with clinical time courses of *BCR-ABL1/ABL1* ratios measured in peripheral blood, we calculate the simulated *BCR-ABL1/ABL1* ratio (in %) and its logarithm as

$$RATIO(t) = \frac{Y}{Y + 2(K_Y - Y)} \cdot 100 \,,$$
$$LRATIO(t) = log_{10}\left(\frac{Y}{Y + 2(K_Y - Y)} \cdot 100\right)$$

thereby accounting for the presence of both *BCR-ABL1* and *ABL1* transcripts in leukemic cells. Herein the carrying capacity K_Y is set to 10^6. Within the model, we define molecular recurrence as the first time point at which RATIO increases above 0.1% and remains there for at least one month.

We obtain patient-specific optimal parameter choices for the model parameters (termed λ) by applying an optimization routine that minimizes the distance between each clinical time course and the corresponding simulation (see Supplementary Text S3).

Results

Model description of *BCR-ABL1* dynamics during dose reduction and after TKI cessation

We analyzed a cohort of 67 patients from the DESTINY trial (NCT01804985 (27), for which complete time course information, i.e. *BCR-ABL1* measurements during the initial treatment response, during the 12-month dose reduction period, and after TKI stop (*clinical reference data set*, Figure 1A). We hypothesized that these complete time courses reveal patient-specific features with respect to CML progression, TKI response and immunological control mechanisms. In order to quantitatively address these aspects, we applied an established mathematical model of CML treatment (12, 13, 31), which explicitly considers interactions between leukemic cells and the immune system (Figure 1B, see Methods) (29).

Figure 2 illustrates the general approach to obtain individual choices of model parameters to fit a patient's time course. We applied a genetic algorithm to optimize the following seven critical parameters: the transition rates between quiescent and active LSC, p_{XY} and p_{YX}, the proliferation rate of active LSC p_Y, the TKI-dependent kill effect *TKI*, immune parameters p_Z and K_Z, and the initial *BCR-ABL1/ABL1* ratio on the log-scale (denoted as *initLRATIO)*. While we cannot be sure whether a

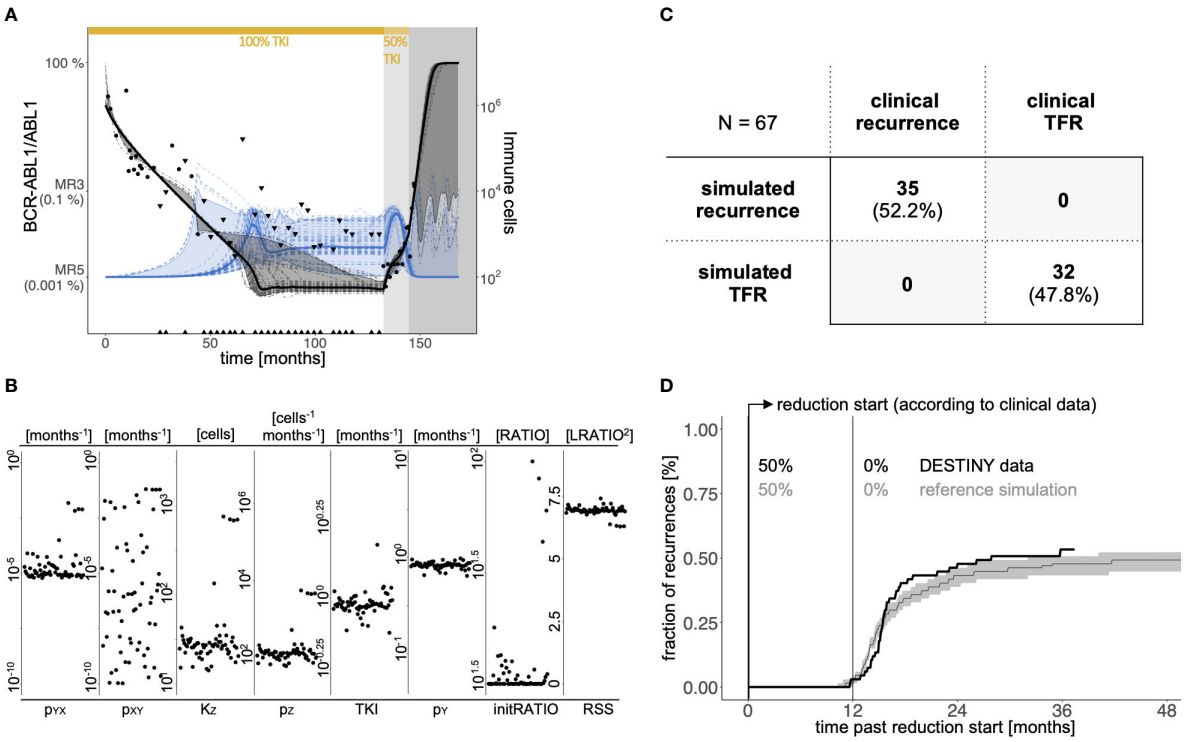


FIGURE 2
(A) Example of clinical response data and model simulations of a patient with CML recurrence after therapy stop. Black dots indicate BCR-ABL1/ABL1-measurements; black triangles represent the quantification limit for undetectable BCR-ABL1 levels (see Supplementary Text S1). White background indicates full dose TKI treatment, light grey indicates the reduction period (50% of full dose), and dark grey refers to the time period after treatment cessation. The bold black line indicates the median and the dark grey ribbon corresponds to the 95% interval of all 64 simulated BCR-ABL1/ABL1-time courses (dashed dark grey lines). The bold blue line indicates the corresponding median of all 64 simulated immune cell counts (dashed blue lines) with the light blue region corresponding to the 95% interval. **(B)** Distribution of individual parameter values of the 64 parameter sets of the same patient as in A, shown separately for the 7 parameters along with the residual sum of squares (RSS) as a measure of fitting quality. The x-axis within each parameter plot reflects the sequence of fits. **(C)** Comparison of the simulated results and the clinical outcomes with respect to TFR or disease recurrence according to a binary classification. Herein we define *simulated recurrence patient* if at least one of the 64 patient-specific parameter sets λ_{ij} resulted in a simulation with molecular recurrence during the follow-up period, while this is not the case for a patient classified as *simulated TFR*. **(D)** Comparison of the time and fraction of recurrences between the patient data according to DESTINY (black line) and the reference simulation (grey). To obtain the curve for the reference simulation, we randomly choose one eligible parameter set λ_{ij} per each patient *j* and calculate whether and when there will be a recurrence in the corresponding simulation. We repeat this sampling approach 1000 times to obtain the median (grey line) and a 95%-confidence region (grey shaded). Time = 0 indicates the start of TKI dose reduction, which corresponds to the time point provided in the clinical data for each patient considered.

unique global optimum is identifiable, we observed that the measurement uncertainties allowed for many reasonable fits. For these reasons we took a complementary approach in which we explicitly consider the intrinsic heterogeneity of the model solutions. Technically, we used 64 independent optimization runs ($i \in \{1 \ldots 64\}$) per patient j, and obtained 64 parameter sets λ_{ij} that reflect the patient-specific variability within the parameter space (Figure 2A, Supplementary Text S3).

Figure 2B indicates the parameter choices for the particular example, while the final column depicts the residual error for each λ_{ij}. Within the figure there are broader regions for some parameter choices (like the immune parameters p_Z and K_Z), although the residual error remains in the same order of magnitude. This confirms the visual impression from Figure 2A that all 64 fits sufficiently explain the given patient time course (see Supplementary Figure S2 for further examples). While 64 fits are chosen as a convenient representation for the intrinsic heterogeneity, increasing this number leads to close to identical results. Application of this optimization approach reassures us that all generic features of CML specific time courses, such as the initial biphasic response, sustained remission or recurrence can be reflected by our mathematical model.

Comparison of recurrence between model simulation and clinical data

Molecular recurrence within the DESTINY trial is defined as two consecutive *BCR-ABL1/ABL1* measurements which exceed 0.1% (MR3). In the model, we mirror this as sustained levels of leukemic cells above 0.1% for at least one month. Applying this definition to the simulations for each of the 64 parameter sets λ_{ij} for all 67 patients, we observed 32 patients with no indication of recurrence, while 35 have several simulations predicting recurrence (including 11 with mixed outcomes and 24 with recurrence predictions only). Viewing this question as a binary classification problem, we applied a receiver operating characteristic (32) analysis to compare the simulated recurrences with true remission status from the *clinical reference data set* (Supplementary Figure S3). We obtained the best correspondence when the patients were classified as *simulated recurrence patient* if at least one of the 64 patient-specific parameter sets λ_{ij} resulted in a simulation with molecular recurrence during the follow-up period. Patients with only non-recurrence simulations were classified as *simulated TFR patients*. With this binary classification the model correctly reproduces the clinical outcome for all patients (Figure 2C).

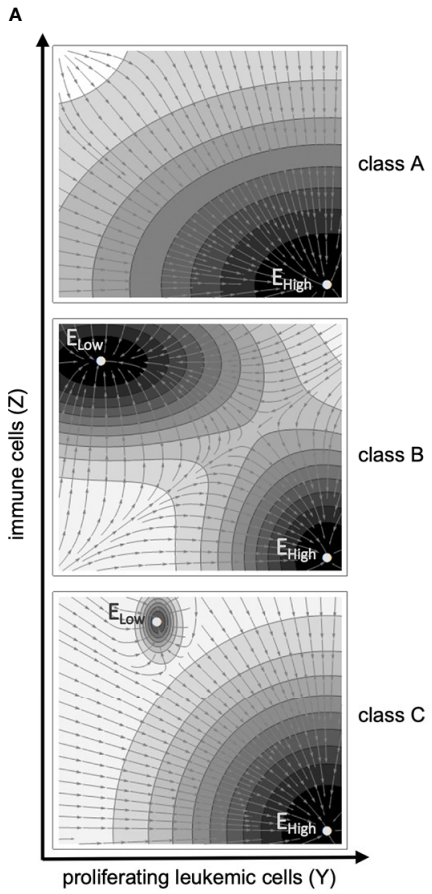
In a complementary approach, we also include the timing of molecular recurrence events to compare our simulations with the *clinical reference data set* from the DESTINY trial. In order to account for the intrinsic heterogeneity of the model solutions we use a sampling approach in which for all 67 patients one of their eligible parameter set λ_{ij} is randomly chosen, while this whole process is repeated 1000 times (see Supplementary Text S4). We obtain the cumulative, time dependent incidence of recurrences as the median and the 95% range over the simulation results from all the repeated realizations. Figure 2D indicates that the model simulations well reflect the timing of molecular recurrences with a prominent increase within a few months after stopping TKI. The fraction of recurrences is slightly underestimated compared to the binary classification in Figure 2C as the repeated sampling strategy rather provides an average recurrence behavior per patient. We point out that for these reference simulations we are using the same time from TKI start until dose reduction as it is denoted for each individual patient in the DESTINY trial (on average 85.4 months).

Our results show that the suggested model is capable of reproducing *BCR-ABL1/ABL1* time courses of CML patients as well as the timing and occurrence of clinically observed recurrences. However, applying the same approach to pre-cessation data only, we show that the model cannot reliably predict the future remission status of a patient (Supplementary Figure S4, Supplementary Text S5). This is particularly true for patients with non- or slowly-increasing *BCR-ABL1* levels during dose reduction as an inference of functional immunological control cannot be obtained with sufficient precision. These findings complement a previous statistical analysis of the DESTINY data that obtained similar results (28).

Immune landscapes account for within-patient heterogeneity

We have previously shown that the mathematical model implies different 'immunological landscapes' characterized by the existence or absence of typical steady states that can be obtained after therapy stop (25, 29). The occurrence, the number, and the status of these steady states are determined by the chosen model parameters λ and are, therefore, specific for each parameter fit (Figure 3A, see Supplementary Text S6). In brief, parameter sets belonging to *class A* are characterized by an insufficient immune response such that there is only one stable steady state describing leukemic dominance (E_{High}). *Class B* refers to scenarios with a strong immune system in which the steady state for leukemic dominance (E_{High}, similar to class A) is accompanied by a second steady state describing immune control of a sufficiently few leukemic cells (remission steady state, E_{Low}). *Class C* presents with a similar immunological landscape as class B while the size of the remission steady state (E_{Low}) is smaller and more difficult to achieve.

As we have not obtained a uniquely identifiable parameter set for all patients j, but use a spectrum of 64 optimization runs i, optimal parameter sets λ_{ij} of the same patient may be classified to different immunological classes A, B or C. Practically, each of the 64 optimal parameter configurations λ_{ij} for a particular patient j, obtained from fitting the complete data set (compare Figure 2A, B), corresponds to one of the three general immune classes A, B or C (Figure 3A). We observed 9 patients for which all the 64 fits were



B

clinical outcome / immune class	clinical recurrence	clinical TFR
B *(N=9)*	1	8
C *(N=9)*	9	0
B/C *(N=35)*	11	24
A/C *(N = 1)*	1	0
A/B/C *(N = 13)*	13	0

FIGURE 3
(A) Sketch of the prototypical immunological landscape classes A, B and C (see Supplementary Text S6). The phase space is spanned by the number of proliferating leukemic cells *Y* on the x-axis and the number of immune cells *Z* on the y-axis. Steady states of the dynamical system are identified by E_{High} and E_{Low} (see Supplementary Text S6). The vector field indicates the direction of model trajectories after TKI cessation and the basin of attractions of the stable steady states. **(B)** Classification of the 67 patients according to immune classes and their clinical outcomes after treatment cessation.

consistently identified as class B and 9 patients for which all fits were consistently identified as class C (Figure 3B, Supplementary Figure S2). However, there are 35 patients for which some parameter configurations indicated class B while others indicated class C (termed B/C) and one patient for which some parameter configurations indicated class A and others class C (termed A/C). The same is true for 13 patients in which all three classes A, B and C were identified (termed A/B/C). Interestingly, class B patients were predominantly those achieving sustained TFR, while all of the class C patients developed molecular recurrence. The same is true for A/C and A/B/C patients, who all fail to achieve sustained TFR. B/C patients appear in both the molecular recurrence and in the sustained TFR groups. We will further analyze to which extend this intrinsic heterogeneity can explain how changes in the dose reduction schedule influence TFR success on the population level.

Treatment duration determines overall TFR success

As a reliable prediction of the remission behavior for individual patients is limited by the insufficiency to infer immunological control parameters prior to TKI stopping (see Supplementary Text S5 and Supplementary Figure S4), we aimed to investigate how general treatment schemes can be optimized such that they are applicable to all patients without prior stratification while maximizing TFR success and reducing TKI usage. We approach this question by comparing the temporal recurrence behavior (compare Figure 2D) and the average total TKI consumption for a range of systematically modified treatment schedules that are applied to the optimal patient parameterization obtained from the *clinical reference data set*.

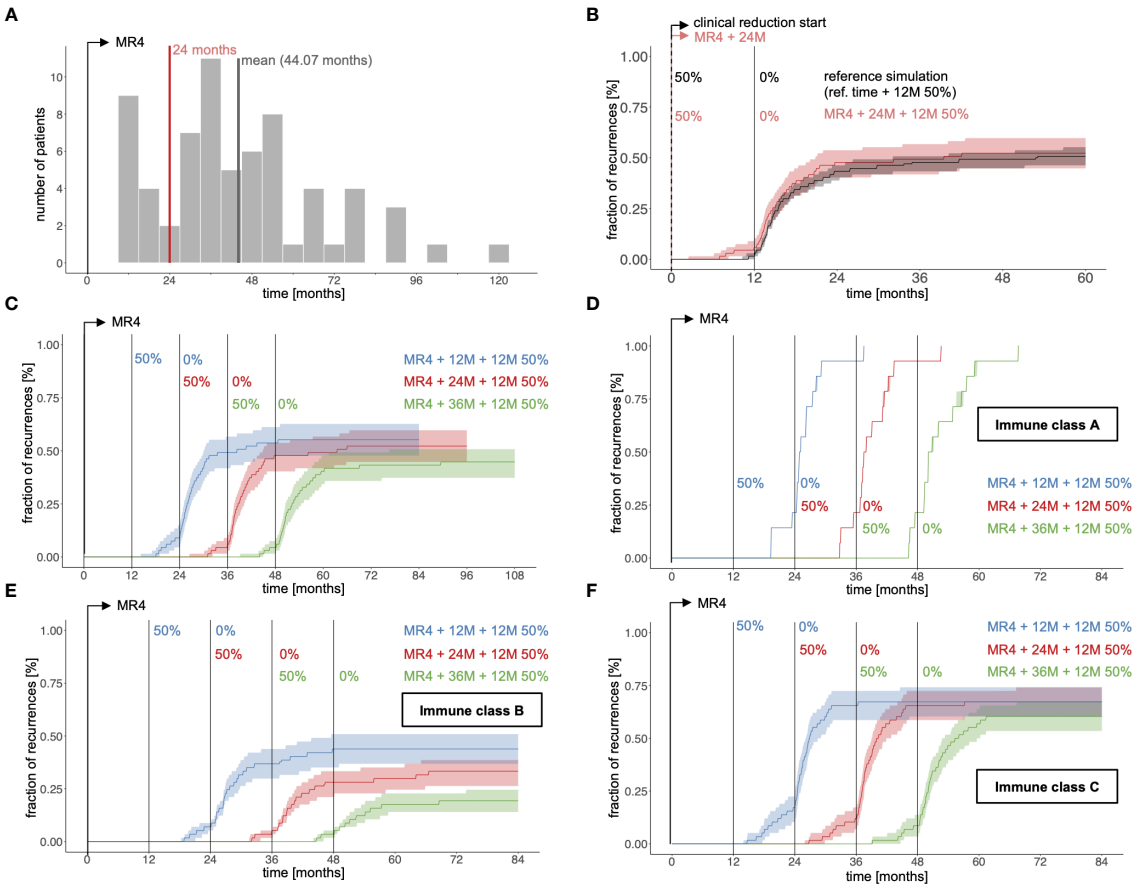


FIGURE 4
(A) Histogram indicating the average time span per patient between reaching MR4 (time = 0) and start of TKI dose reduction for the clinical reference data from the DESTINY trial (grey bars) compared to the MR4+24M+12M50% scenarios in which dose reduction is initiated precisely 24 months after the corresponding fits reached MR4 (red line). **(B)** Fraction of recurrences as a function of time comparing the reference simulation (grey; simulations according to the clinical reference data with respect to time point of dose reduction) and a scenario with a 12 month dose reduction period initiated 24 months after the simulated time courses have reached MR4 (red, indicated as MR4+24M+12M50%). Time = 0 corresponds to the start of TKI dose reduction, either according to the clinical data or of 24 months after reaching MR4. **(C)** Same analysis in which the time between reaching MR4 and the initiation of the 12 month dose reduction period varies from 12 to 36 months (indicated as MR4+12M+12M50%, MR4+24M+12M50%, MR4+36M+12M50%). Time = 0 corresponds to the time point of reaching MR4 for each simulation. **(D-F)** Corresponding simulations to subfigure C, stratified according to the immune class for each optimal parameter set λ_{ij}. The subfigures contain only patients j with at least one optimal parameter set λ_{ij} classified to either immune class A, B or C, while the sampling only considers parameter sets λ_{ij} that correspond to the respective immune class.

As a first step we used our computational model to simulate the 12 month dose reduction period starting strictly 24 months past reaching the 0.01% (MR4) remission level (MR4+24M+12M50% scenario) for all 67 patients instead of using the observed time points from the *clinical reference data set* (Figure 4A). Overall, the simulated CML recurrence for the amended treatment scheme is similar to that observed for the clinically reference simulation (Figure 4B). However, for the amended scenario, TKI is only administered for 24 months past reaching MR4, while the drug is given on average for 44.1 months after MR4 for the reference simulations (Figure 4A).

Varying the full dose TKI treatment time between 12 and 36 months past reaching MR4 (Figure 4C) clearly demonstrates that shorter TKI duration leads to more recurrences, while longer TKI treatment improves TFR success. The quantitative increase in TFR success per additional year of TKI treatment compares well with findings from the EURO-SKI trial on TKI discontinuation (8). To further examine how treatment duration acts on the different immune classes, we stratified the patients as to whether any of their optimal parameter sets λ_{ij} belong to class A, B or C. Applying the same sampling approach as above (Supplementary Text S4) separately to the eligible parameter sets of those subcohorts (Figure 4D-F) indicated that both class B and class C configurations will benefit from longer treatments, although the benefit for class C appears marginal. As expected, parameter configurations of class A develop molecular recurrence

irrespective of the treatment configuration. Only patients that can in principle establish immune surveillance of their residual leukemia levels may therefore benefit from longer treatment durations.

TKI dose reduction strategies to optimize TFR success

To address the effect of TKI dose on TFR success we considered a situation where simulated patients receive 36 months full dose treatment past reaching MR4 (MR4 + 36M) and compared it to the reduction scheme discussed above, in which a 24 month full dose treatment after reaching MR4 precedes a 12 month reduction to either 50% of the original dose (MR4 + 24M + 12M50%) or to 25% of the original dose (MR4 + 24M + 12M25%). While some patients in the latter scenarios present with disease recurrence earlier than they would do with the full dose treatment, those patients are likely to fail anyway. Figure 5A indicates that those three scenarios can hardly be distinguished with respect to their long-term outcome, thereby suggesting that overall treatment duration determines the success rate, while there is potential to achieve the same results with substantially less TKI.

To systematically analyze this, we simulated further reduction schemes, all extending over 36 months past reaching MR4. Earlier initiation of dose reduction at 12 months past reaching MR4 leads to a similar fraction of recurrences compared to the above scenarios (MR4+12M+24M50% and MR4+12M+24M25%, Figure 5B). However, this effect is lost if the reduction step is initiated right after reaching MR4 (MR4+36M50% and MR4+36M25%, Figure 5C) resulting in an increase of the overall proportion of recurrences.

Complementary to this observation it is interesting that a stepwise dose reduction to 50% initially and 25% thereafter (MR4 +12M+12M50%+12M25%) hints towards a lower recurrence rate (Figure 5D), while requiring only 58% of the TKI amount administered past MR4 compared to the 36M full dose scenario. Again, an earlier introduction of the reduction regimen right after reaching MR4 (MR4+18M50%+18M25%) seems to oppose this effect. Having a closer look at the MR4+12M+12M50%+12M25%

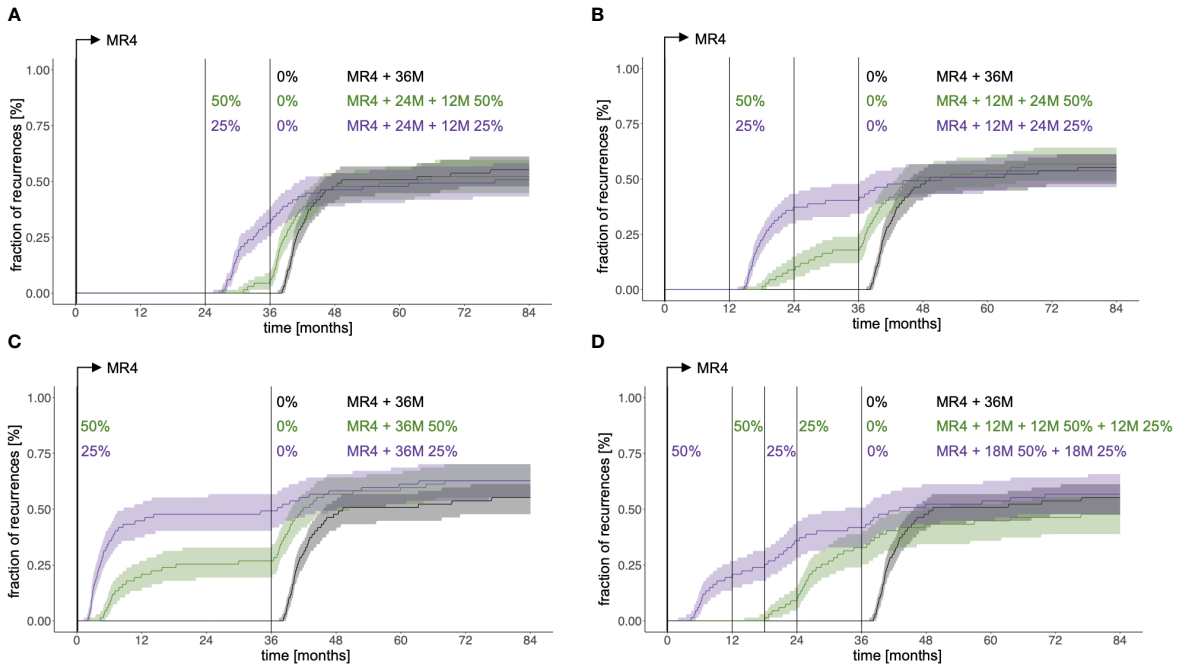


FIGURE 5
Fraction of recurrences as a function of time comparing immediate therapy cessation 36 months after reaching MR4 (black) with different scenarios of dose reduction covering the same overall treatment duration: **(A)** 24 months full dose treatment past reaching MR4 plus 12 month dose reduction to 50% of the initial dose (green, indicated as MR4+24M+12M50%) and 24 months full dose treatment past reaching MR4 plus 12 month dose reduction to 25% of the initial dose (purple, indicated as MR4+24M+12M25%). **(B)** 12 months full dose treatment past reaching MR4 plus 24 month dose reduction to 50% of the initial dose (green, indicated as MR4+12M+24M50%) and 12 months full dose treatment past reaching MR4 plus 24 month dose reduction to 25% of the initial dose (purple, indicated as MR4+12M+24M25%). **(C)** dose reduction to 50% of the initial dose for 36 months immediately after reaching MR4 (green, indicated as MR4+36M50%) and dose reduction to 25% of the initial dose for 36 months immediately after reaching MR4 (purple, indicated as MR4+36M25%). **(D)** 12 months full dose treatment past reaching MR4 plus 12 month dose reduction to 50% of the initial dose plus 12 month dose reduction to 25% of the initial dose (green, indicated as MR4+12M +12M50%+12M 25%) and dose reduction to 50% of the initial dose for 18 months immediately after reaching MR4 plus 18 month dose reduction to 25% of the initial dose (purple, indicated as MR4+18M50%+18M25%).

scenario we speculate that the advantage results from a better convergence of the immune class C patients to the remission steady state while this aim is not yet reached for patients of the immune class B (Supplementary Figure S5). For class C patients, full dose treatment may actually result in overtreatment and a deactivation of the immune system, while this effect is prevented by the stepwise reduction of TKI dose (Supplementary Figure S6). Our overall conclusions do not change if e.g. an overall treatment duration of 48 months post reaching MR4 is considered (Supplementary Figures S7).

Discussion

Our modeling results confirm clinical findings that the overall time of TKI treatment is a major determinant of TFR success (8), but at the same time indicate that lower dose TKI treatment may be sufficient to achieve the same results for many patients. Having a more detailed look on the response dynamics during dose reduction and after treatment cessation, we reason that a subset of patients with presumably insufficient immune control will inevitably relapse, no matter whether TKI is stopped all at once or reduced in a stepwise manner. During dose reduction, such patients often present with substantially increasing *BCR-ABL1* levels. However, in most patients there is no or only a mild increase of *BCR-ABL1* levels during TKI dose reduction, for which reliable predictions of TFR success after stopping treatment cannot be drawn (see also (28)). Our modeling results indicate that a stepwise dose reduction prior to TKI cessation does not limit the overall success rate of TFR for this patient group, while it can already substantially reduce TKI associated side effects (27, 33) as well as overall treatment costs.

The importance of the immune system in the sustained control of residual disease levels is widely recognized and many immunologically relevant subpopulations and their interactions are studied in the context of CML recurrence (21–23). While promising correlations, e.g. with the abundance of $CD56^{dim}$ natural killer cells (18) or activated $CD86^{+}$ plasmacytoid dendritic cells (19) have been identified, no unique marker nor control mechanism has been identified that allows a reliable prediction of TFR success. This is also supported by our dynamical analysis of CML time course data, which allows the identification of patients at high risk of molecular recurrence presenting with substantially increasing *BCR-ABL1* levels after TKI dose reduction but performs insufficiently to discriminate patients presenting with no or a mild increase of *BCR-ABL1* levels. However, our simulation approach allows us to study how an artificial, but individually parameterized patient cohort behaves for modified dose reduction schemes that can be applied to an unstratified patient cohort.

We suggested previously that the long-term response of CML patients is not limited by the TKI drug efficiency but by the rare activation of leukemic stem cells (31), which allows for lower TKI doses in maintenance therapy. In the context of the current simulation approach, we systematically explored the potential of TKI dose reduction beyond that used in the DESTINY trial, also with respect to TKI cessation. While we demonstrated that the initiation of 50% dose reduction 24 months after reaching MR4 closely mimics the heterogeneous timing of dose reductions from the DESTINY patients, we also explored more sophisticated dose reduction approaches. In particular, we observed that dose reductions to 25% of the original TKI dose also lead to comparable results. This finding opens the possibility to initiate two-step reduction schemes that can further broaden the range of possible treatment options. Our simulations suggest that halving the dose twice in annual intervals one year after reaching MR4 (MR4 + 12M + 12M50% + 12M25%) could perform at least equally well compared to a full dose treatment with TKI over the whole three year period. We are looking forward to a recently initiated clinical trial investigating this question (32).

A range of clinical studies that administer TKI at lower than the standard dose underline the potential of these suggestions. Several studies, especially on second generation TKI in newly diagnosed patients, document the clinical efficacy of such reduced dose regimens to achieve cytogenetic and molecular remission while at the same time delivering an improved side effect profile (34–37). Other approaches use dose reduction for patients in major or deep molecular remission to manage TKI-related side effects and to improve the patients' quality of life (38, 39). The results indicate that TKI dose reduction is safe, has no effect on long term outcome and only minimal effects on cytogenetic and molecular response (40). These clinical results are complemented by earlier mathematical modelling approaches of our group (31). In this work we could show that the cytotoxic effect of TKIs is limited by the rare activation of leukemic cells, thereby reasoning that higher than necessary TKI doses do not confer an additional benefit.

Our modeling analysis along with the overall results of the DESTINY trial (27, 33) suggest that TKI dose reductions prior to stopping maintain TKI-based control of residual disease levels and may further confer an additional advantage with respect to TFR success compared to immediate TKI cessation. We hypothesize that this beneficial effect results from sensitization of the immune system prior to stopping. While it is beyond the scope of this work, these results stimulate speculations on the extent to which a mild increase in leukemic cell load under reduced TKI dose could be a useful trigger for immunological control mechanisms prior to stopping TKI. Further approaches to address this question may include vaccination strategies applied during deep remission (41) and before the patients discontinue therapy.

While both the small number of clinically available reference data sets and the simplifying assumptions underlying our modeling approach limit the generalizability of our results, we aimed for a strong internal consistency. To this end we used internal controls, such as the variation of overall TKI treatment durations, and

compared them to clinical findings. Our results are, therefore, generic in nature but demonstrate a strong side of this systems biological approach: to underpin conceptual consideration with quantitative arguments that can guide the planning of experimental and clinical studies. For the particular situation, we argue that the clinical potential for TKI dose reductions in CML patients with sustained remission is not exhausted and may not compromise the goal of complete TKI cessation.

Data availability statement

The data used in this study is available upon request from the corresponding author. Requests to access these datasets should be directed to Ingmar Glauche, ingmar.glauche@tu-dresden.de.

Ethics statement

The DESTINY trial ((27), NCT 01804985) was conducted in accordance with the Declaration of Helsinki and applicable regulatory requirements. The protocol was approved by the North West - Liverpool East Committee of the UK National Research Ethics Service. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conception and design: EK, CB, IR, AF, IG. *Development of methodology:* EK, CB, TZ, AF, IG. *Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):* RC. *Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):* EK, CB, AF, IG. *Writing, review, and/or revision of the manuscript:* EK, CB, TZ, RC, IR, AF, IG. *Study supervision:* IR, AF, IG. All authors contributed to the article and approved the submitted version.

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Conflict of interest

RC is a consultant for Pfizer. IR reports receiving a commercial research grant from Bristol-Myers Squibb and has received speakers bureau honoraria from Bristol-Myers Squibb and Janssen-Cilag. IG reports receiving a commercial research grant from Bristol-Myers Squibb and from GlaxoSmithKline.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1028871/full#supplementary-material

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Successful treatment discontinuation in CML patients with full-dose and low-dose TKI: Results from real-world practice

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Background: In clinical studies, some patients who achieve deep molecular response (DMR) can successfully discontinue tyrosine kinase inhibitor (TKI). TKI dose reduction is also an important aspect of alleviating adverse effects and improving quality of life. This study aimed to explore the outcome after drug withdrawal in Chinese CML patients.

Methods: We conducted a retrospective analysis of the outcome of 190 patients who stopped TKI. 27 patients experienced dose reduction before TKI discontinuation. The median duration of TKI treatment and MR4 before discontinuation was 82 months and 61 months.

Results: With median follow-up after stopping TKI treatment of 17 months, the estimated TFR (Treatment Free Remission) were 76.9% (95%CI, 70.2%–82.4%), 68.8% (95%CI, 61.3%–75.2%), and 65.5% (95%CI, 57.4%–72.5%) at 6, 12 and 24 months. For full-dose and low-dose TKI groups, the TFR at 24 months was 66.7% and 55.8% (p = 0.320, log-rank). Most patients (56/57) quickly achieved MMR after restarting TKI treatment. Multivariable analysis showed that patients with TKI resistance had a higher risk of molecular relapse than patients without TKI resistance ($p < 0.001$).

Conclusion: TFR rates were not impaired in patients experiencing dose reduction before TKI discontinuation compared to patients with full-dose TKI. Our data on Chinese population may provide a basis for the safety and feasibility of TKI discontinuation, including discontinuation after dose reduction, in clinical practice.

KEYWORDS

chronic myeloid leukemia, tyrosine kinase inhibitor, dose reduction, treatment free remission, real-world practice

1 Introduction

The advent of tyrosine kinase inhibitor (TKI) has revolutionized the treatment of chronic myeloid leukemia (CML) and improved the prognosis of patients to near-normal levels (Sasaki et al., 2015). However, the adverse effects associated with long-term TKI therapy are an overwhelming factor affecting the quality of life (Steegmann et al., 2016). Besides, the high cost of persistent TKI treatment is increasingly becoming an important issue (Phuar et al., 2019). Over recent years, numerous clinical studies have proved that about 40%–60% of patients with

deep molecular response (DMR) could achieve TFR (Rea et al., 2017b; Etienne et al., 2017; Mahon et al., 2018; Saussele et al., 2018; Nagafuji et al., 2019; Kimura et al., 2020; Shah et al., 2020). In this context, treatment-free remission (TFR) is a major goal for all CML patients according to the current guidelines (Hochhaus et al., 2020). Recently, The Gruppo Italiano Malattie EMatologiche dell'Adulto (GIMEMA; Italian Group for Hematologic Diseases of the Adult) CML Working Party developed a project designed to select treatment policies that might increase the likelihood of achieving TFR. A consensus was reached on disease risk assessment, first-line treatment, and the responses that require a change of the TKI, contributing to optimizing the treatment strategy for TFR (Baccarani et al., 2019). However, data on the impact and outcomes of TKI discontinuation outside of clinical studies remain sparse. In clinical practice, TKI dose reduction is also an important aspect of alleviating adverse effects and improving quality of life (Russo et al., 2015; Iurlo et al., 2020). In addition, several studies have shown that low-dose TKI treatment is effective in maintaining major molecular response (MMR) (Rea et al., 2017a; Clark et al., 2017; Claudiani et al., 2021). Moreover, dose-halving therapy yields higher molecular recurrence-free survival rate compared to direct TKI discontinuation (Luo et al., 2022). Our recent study also confirmed the feasibility of TKI dose reduction therapy for Chinese CML patients (Chen et al., 2022). The concern of whether dose reduction before TKI discontinuation affects patient's access to TFR is also of interest to investigators. The DESTINY study has investigated the effects of low-dose TKI treatment withdrawal before stopping TKI, of which patients with MMR at dose reduction experienced a TFR rate of 36% at 36 months and patients with DMR reported a TFR rate of 72% (Clark et al., 2019), indicating TKI de-escalation is safe for most patients with DMR. TKI de-escalation - to discontinue TKI may be an alternative way to achieve TFR (Kunbaz and Eskazan, 2019). Still, more studies are needed to investigate the possibility of TKI discontinuation in patients with dose reduction, prior treatment failure, or other problems in the real world. In this study, we retrospectively analyzed a real-world series of patients with CML who discontinued TKI, including patients who received low-dose TKI therapy prior to discontinuation.

2 Materials and methods

2.1 Patients

We collected data from CML-CP patients who discontinued TKI between June 2013 and July 2022 from the three hospitals in China (Union Hospital, Tongji Medical College, Huazhong University of Science and Technology; The Affiliated Cancer Hospital of Zhengzhou University; Yichang Central People's Hospital & First Clinical Medical College of China Three Gorges University). All patients were diagnosed with CML-CP by bone marrow cell morphology, cytogenetics, and molecular biology examination. All patients who discontinued TKI were treated with TKI for at least 3 years, and MR4 lasted at least 2 years.

2.2 Molecular response definitions

Molecular monitoring was assessed by quantitative polymerase chain reaction (qPCR). The *BCR-ABL 1* level was expressed on the international scale (IS). The Molecular response was assessed according to ELN criteria (Hochhaus et al., 2020). In detail, MMR was defined as a *BCR-ABL/ABL* ratio ≤0.1%, and MR4 was defined as a *BCR-ABL/ABL* ratio ≤0.01%. Molecular relapse was defined as loss of MMR. TFR was calculated from TKI discontinuation to the loss of MMR.

2.3 Statistical analysis

Comparison of continuous variables was tested by Student t-test or Mann-Whitney *U* test, and comparison of categorical variables was tested by χ^2 or Fisher exact test. TFR was estimated using the Kaplan-Meier method, and comparison between groups was used log-rank tests. Univariate analysis of potential clinical factors associated with molecular relapse was performed using the Kaplan-Meier method. Multivariate analysis was performed using the Cox regression analysis. Proportionality hazards assumptions were checked for factors included in Cox regression analysis. All analyses were carried out using SPSS, version 22 statistical software, and GraphPad Prism 7.

3 Result

3.1 Patient characteristics

Among 1476 CML patients, 190 patients who discontinued TKI were included in this study, 97 of whom were female and 93 were male, the median age was 43 years (range, 3–74 years) (Figure 1, Table 1). 182 patients were on imatinib at diagnosis, of which 81 patients switched to second-generation TKI for resistant or non-resistant reasons. Eight patients were on a second-generation TKI (2G-TKI) at diagnosis. 101 patients took imatinib, 15 patients took dasatinib, 73 patients took nilotinib and 1 patient took Flumatinib at discontinuation, respectively. The median duration of TKI treatment before discontinuation was 82 months (range, 36–222), the duration of MR4 before TKI discontinuation was 61 months (range, 24–162), and the time to MR4 was 12 months (range, 3–112). Twenty seven patients underwent dose reductions before TKI discontinuation. Forty eight patients experienced TKI resistance (imatinib, $n = 48$) before TKI discontinuation according to ELN criteria (Hochhaus et al., 2020).

3.2 Low-dose TKI treatment

In clinical practice, TKI dose reduction is also an important aspect of alleviating adverse effects and improving quality of life. Twenty seven patients underwent dose reductions before TKI discontinuation for adverse events ($n = 17$), desire to stop TKI ($n = 8$), and finical burden ($n = 2$) (Table 2). The median duration of low-dose TKI treatment was 18 months (range, 3–72). Overall, 22 patients experienced half of the standard-dose treatment. All 27 patients were MR4 or deeper at the time of TKI discontinuation. The median duration of TKI treatment before discontinuation was 83 months (range, 43–162), duration of MR4 before TKI discontinuation was 58 months (range, 24–159), and the time to MR4 was 17 months (range, 3–90) (Table 2).

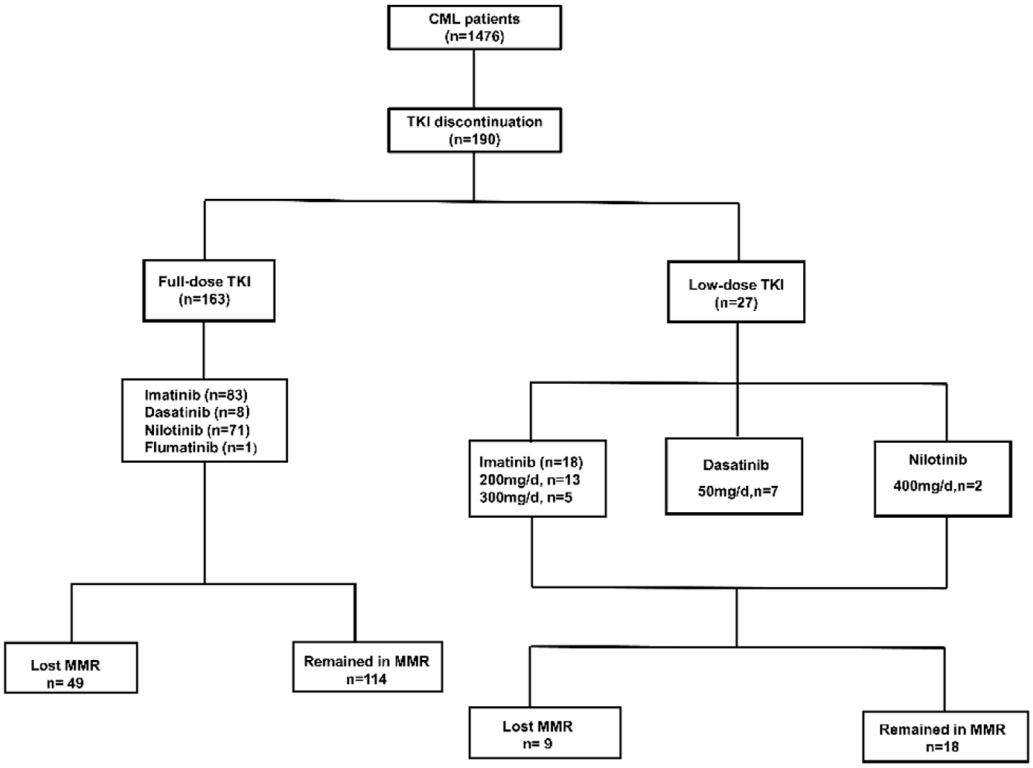

FIGURE 1
Study population flowchart. TKI, tyrosine kinase inhibitor.

3.3 Discontinuation in patients with full-dose and low-dose TKI

With median follow-up after stopping TKI treatment of 17 months (range, 2–96 months), 58 patients lost MMR, nine patients lost MR4, and 123 patients remained MR4 or deeper. The estimated overall TFR were 76.9% (95%CI, 70.2%–82.4%), 68.8% (95% CI, 61.3%–75.2%), and 65.5% (95%CI, 57.4%–72.5%) at 6, 12 and 24 months (Figure 2A). For full-dose and low-dose TKI groups, the TFR at 6 months were 78.4% (95%CI, 71.3%–84.0%) and 63.8% (95% CI, 39.6%–80.4%), the TFR at 12 months were 70.3% (95%CI, 62.3%–76.9%) and 55.8% (95% CI, 30.3%–75.2%), the TFR at 24 months were 66.7% (95%CI, 58.0%- 74.0%) and 55.8% (95% CI, 30.3%–75.2%) (Figure 2B). Hence, patients with low-dose TKI before TKI discontinuation achieved TFR rates similar to those of patients treated with maintenance full-dose TKI. Moreover, 81.5% (22/27) of low-dose patients experienced half of the standard-dose treatment. Those patients experienced a similar TFR rate compared to patients with less than 50% reduction in TKI ($p = 0.242$, log-rank) (Figure 2C). Moreover, we found that patients with at least 18 months of dose reduction achieved a similar TFR rate to those with less than 18 months of dose reduction ($p = 0.172$, log-rank) (Figure 2D). Our data showed that TKI de-escalation did not impact TFR.

3.4 Predictive factors for molecular relapse

Univariate analysis showed that the type of TKI at discontinuation, TKI resistance, line of therapy, and time to MR4 was the clinical variable significantly associated with molecular recurrence (Table 3; Figure 3). Multivariate analysis showed that only a history of TKI resistance was significantly associated with molecular recurrence. We performed a subgroup analysis to compare the differences in TFR among patients with different TKI treatments. We divided the patients into five groups: patients with imatinib-resistant who switched to 2G-TKI therapy ($n = 39$) or not ($n = 9$), patients without imatinib-resistant who switched to 2G-TKI therapy ($n = 42$) or not ($n = 92$) and patients treated with 2G-TKI in the first line ($n = 8$). For patients with imatinib resistance, those who switched to 2G-TKI therapy had remarkably higher TFR rates than those who did not ($p < 0.001$, log-rank) (Figure 4A). Similarly, patients without resistant who switched to 2G-TKI experienced higher TFR rates than those on imatinib treatment ($p < 0.001$, log-rank) (Figure 4B). We also found patients who switched to 2G-TKI therapy for imatinib resistance or non-resistance reasons achieved similar TFR rates compared to patients treated with 2G-TKI in the first line (Figures 4C, D). These results indicated that 2G-TKI therapy may yield higher TFR rates than imatinib in both imatinib-resistant and non-imatinib-resistant patients. In addition, TKI discontinuation should be attempted with great prudence in imatinib-resistant patients who maintaining imatinib therapy.

3.4.1 Outcomes after molecular relapse

In the present study, 58 patients lost MMR at a median time of 4 months (range, 1–23 months), with 42 (72.4%) patients losing MMR within 6 months, 54 (93.1%) patients lost MMR within 12 months. At the last follow-up, 56/57 patients who were restarted TKI regained MMR with a median time of 3 months (range, 1–29), and 47 patients regained MR4 with a median time of 4 months (range, 1–11). For the

TABLE 1 Patient characteristics.

	Full-dose (*n* = 163)	Low-dose (*n* = 27)	Overall (N = 190)	*p*
Median age (range), years	43 (3–74)	49 (5–65)	43 (3–74)	0.387
Male, n (%)	83 (50.9)	14 (51.9)	97 (51.1)	0.929
TKI at TKI discontinuation (n%)				
imatinib	83 (50.9)	18 (66.7)	101 (53.2)	<0.001
dasatinib	8 (4.9)	7 (25.9)	15 (7.9)	
nilotinib	71 (43.6)	2 (7.4)	73 (38.4)	
flumatinib	1 (0.6)	1 (0.5)		
Current TKI therapy line, n (%)				
1st	90 (55.2)	18 (66.7)	109 (57.4)	0.437
2nd	69 (42.3)	9 (33.3)	77 (40.5)	
3rd	4 (2.5)	x	4 (2.1)	
Sokal score, n (%)				0.053
Low	47 (28.8)	5 (18.5)	52 (27.4)	
Intermediate	24 (14.7)	5 (18.5)	29 (15.3)	
High	11 (6.7)	6 (22.2)	17 (8.9)	
NA	81 (49.7)	11 (40.7)	92 (48.4)	
ELTS score, n (%)				0.484
Low	52 (31.9)	9 (33.3)	61 (32.1)	
Intermediate	23 (14.1)	4 (14.8)	27 (14.2)	
High	7 (4.3)	3 (11.1)	10 (5.3)	
NA	81 (49.7)	11 (40.7)	92 (48.4)	
History of TKI resistance, n (%)	40 (24.5)	8 (29.6)	48 (25.3)	0.573
IFN-α before TKI, n (%)	11 (6.7)	3 (11.1)	14 (7.4)	0.422
TKI duration before discontinuation: (range), months	82 (36–222)	83 (43–162)	82 (36–222)	0.887
Time to MR4 (range), months	12 (3–112)	17 (3–90)	12 (3–112)	0.606
MR4 duration before TKI discontinuation (range), months	62 (24–162)	58 (24–159)	61 (24–162)	0.427
Dose reduction n, %			27 (14.2)	

TKI, tyrosine kinase inhibitor.

full-dose group, 49 patients were retreated with TKI (imatinib 400 mg/d, *n* = 27; imatinib 600 mg/d, *n* = 2; imatinib 200 mg/d, *n* = 4; dasatinib 100 mg, *n* = 2; nilotinib 300 mg/d, *n* = 2; nilotinib 600 mg/d, *n* = 9; Flumatinib 600 mg/d, *n* = 3), of which 49 patients achieved MMR and 41 patients achieved MR4. For the low-dose group, eight patients were restarted on imatinib treatment (200 mg/d, *n* = 4; 300 mg/d, *n* = 2, 400 mg/d, *n* = 2) and six of them obtained MR4. At last follow-up, one patient died of non-CML-related disease, one patient was not in MMR, 18 patients were in MMR, and 170 patients were in MR4 or deeper response.

3.4.2 TKI withdrawal syndrome

In this study, 14 patients (7.4%) developed transient musculoskeletal pain within 12 months after TKI discontinuation, of which 10 patients were on imatinib and 4 patients were on nilotinib. 13 patients were in standard-dose group, only one patient was in the de-escalation group. Thirteen patients reported a grade 1–2 TKI withdrawal syndrome and one patient reported a grade 3 TKI withdrawal syndrome. Three patients lost MMR after the occurrence of musculoskeletal pain.

4 Discussion

Currently, long-term TKI treatment is no longer indicated for all patients with CML, as some patients who obtain DMR can safely discontinue TKI. For example, the DASFREE study showed the 1-year TFR rate was 48%, which was 58% in the ENESTop study (Mahon et al., 2018; Shah et al., 2020). The EURO-SKI study with the largest number of patients reported a TFR rate of 61% at 6 months, 50% at 2 years, and 49% at 3 years (Saussele et al., 2018). In addition, several

TABLE 2 Reasons and dose for patients with dose reduction.

Patients no.	MR at time of dose reduction	Reason for dose reduction	Low-dose TKI	Duration of low-dose TKI (month)
1	MR4	leukopenia	Imatinib,300 mg/d	55
2	Not in MMR	leukopenia	Imatinib,300 mg/d	66
3	MR4	edema	Imatinib,200 mg/d	15
4	MR4	edema	Imatinib,200 mg/d	12
5	MR4	finical burden	Imatinib,200 mg/d	33
6	MR4	anorexia	Imatinib,200 mg/d	11
7	MR4	anemia	Imatinib,200 mg/d	12
8	MR4	anemia, fatigue	Imatinib,300 mg/d	39
9	MR4	desire to stop TKI	Imatinib,200 mg/d	23
10	MR4	desire to stop TKI	Imatinib,200 mg/d	3
11	MR4	anemia	Imatinib,200 mg/d	23
12	MR4	edema	Imatinib,200 mg/d	7
13	MR4	elevated bilirubin	Nilotinib, 400 mg/d	24
14	MR4	desire to stop TKI	Dasatinib, 50 mg/d	19
15	MR4	pleural effusion	Dasatinib, 50 mg/d	20
16	MR4	desire to stop TKI	Dasatinib, 50 mg/d	4
17	MR4	desire to stop TKI	Imatinib,200 mg/d	38
18	MR4	pericardial effusion	Nilotinib, 400 mg/d	3
19	MR4	fatigue	Imatinib,200 mg/d	18
20	MR4	desire to stop TKI	Imatinib,200 mg/d	24
21	MR4	pleural effusion	Dasatinib, 50 mg/d	14
22	MR4	pleural effusion	Dasatinib, 50 mg/d	21
23	MR4	finical burden	Dasatinib, 50 mg/d	20
24	MR4	desire to stop TKI	Dasatinib, 50 mg/d	12
25	MR4	desire to stop TKI	Imatinib, 300 mg/d	72
26	MR4	edema	Imatinib, 300 mg/d	3
27	MR4	fatigue	Imatinib, 200 mg/d	17

MR, Molecular response.

results from real-world studies demonstrated the feasibility of TKI discontinuation. An observational study reported an estimated TFR at 12 months of 69% for Italian patients (Fava et al., 2019). A study from a Spanish research group described a TFR rate at 4 years of 64% (Hernandez-Boluda et al., 2018). Recently, a Swedish discontinuation study showed that 62.2% of patients maintained TFR at the last follow-up (Flygt et al., 2021). However, limited data are available on TKI discontinuation for Chinese CML patients. In the present study, a total of 190 patients who discontinued TKI medication were included in this study. The estimated TFR was 68.8%, and 65.5% at 12 and 24 months, comparing favorably to the TFR rate of approximately 50% in most clinical studies (Saussele et al., 2018). The rate is also similar to the TFR rate reported in Italian, Spanish, and Swedish patients (Hernandez-Boluda et al., 2018; Fava et al., 2019; Flygt et al., 2021). After discontinuation, most relapse occurred within the first 6 months. In this study, 72.4% of patients lost MMR within 6 months, and 93.1% of patients lost MMR within 12 months. One patient lost MMR at 23 months after TKI discontinuation. A recent study showed an estimated rate of molecular recurrence after 2 years of discontinuing imatinib of 18% (Rousselot et al., 2020). Hence, prolonged molecular monitoring is still necessary for patients who have not relapsed in the early phase of drug discontinuation. Most patients (56/57) who were restarted TKI regained MMR, and 47 patients regained MR4 with a median time of 4 months (range, 1–11). The results within the Chinese population showed that TKI discontinuation is safe and feasible also outside controlled clinical trials.

At present, dose optimization is increasingly emphasized as an important part of individualized treatment for CML patients with different demands. In the present study, 27 patients experienced

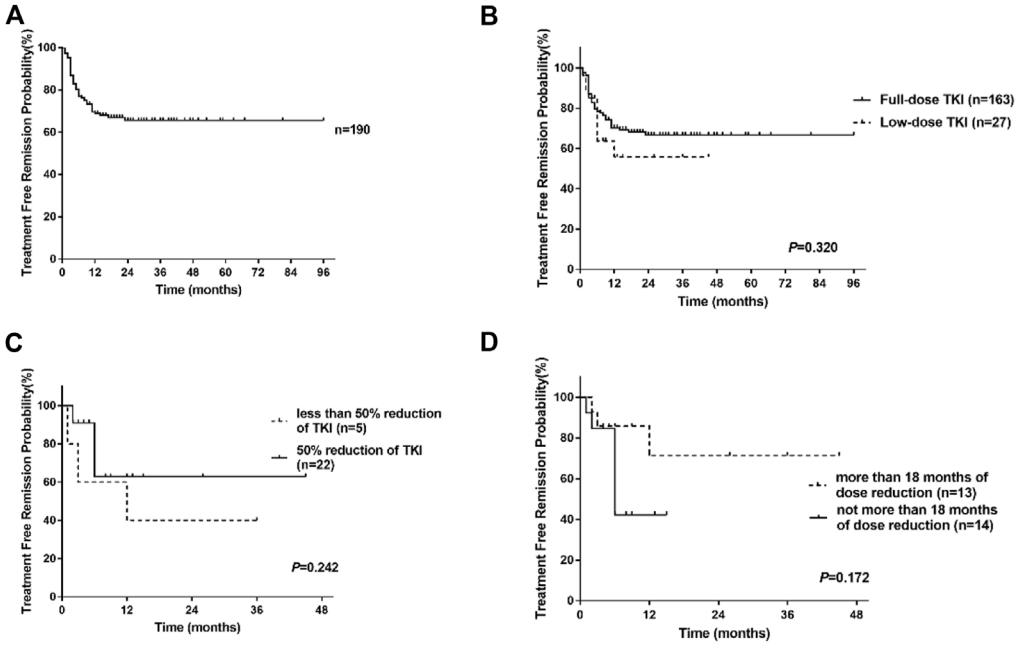

FIGURE 2
Treatment-free remission (TFR) after tyrosine kinase inhibitor (TKI) therapy. **(A)** TFR in the overall patients. **(B)** TFR in patients according to the dose of TKI. **(C)** TFR in patients with low-dose TKI according to the degree of TKI reduction. **(D)** TFR in patients with low-dose TKI according to the duration of dose reduction.

dose reduction before stopping TKI for adverse events (n = 17), desire to stop TKI (n = 8), and finical burden (n = 2). Patients with low-dose TKI treatment had a TFR of 63.8% at 6 months and 55.8% at 12 months. Similarly, patients with full-dose displayed a TFR of 78.4% and 70.3% at 6 months and 12 months, respectively. In the study of Cayssials et al. (2020), the TFR rate at 12 months was 56.8% for full-dose and 80.8% for the low-dose group. However, the DESTINY study reported patients experiencing dose reduction had a TFR rate of 72% after 24 months off TKI (Clark et al., 2019), which appeared to be preferable to the TFR rate of 50% in the EURO-SKI study (Saussele et al., 2018). Claudiani et al. (2021) reported a TFR of 74.1% among patients who have experienced dose reduction. In Claudiani et al. (2021) study, the median duration of MR4 before stopping TKI was 6.1 years, significantly longer than that in the EURO-SKI of 3.1 years. Thus, they suggested that the increased TFR in the low-dose group might be related to the prolonged duration of MR4 to stopping TKI. Recently, Iurlo et al. (2022) investigated the effect of TKI reduction on TFR in a large multicenter cohort of 194 patients with low-dose TKI. Iurlo et al. (2022) study, 71.1% patients were still in TFR after a median follow-up of 29.2 months, which is better than the 62% reported in a recent Italian study with 293 patients who discontinued TKI after a median follow-up of 34 months (Fava et al., 2019; Iurlo et al., 2022). Still, it remains controversial whether the dose reduction before TKI withdrawal can lead to an improvement in TFR. 81.5% (22/27) low-dose patients experienced half of the standard-dose treatment. Moreover, we found the degree of TKI dose reduction was not related to the successful discontinuation, in agreement with the study of Cayssials et al. (2020). Fassoni et al. (2018) developed a mathematical model based on selected patients in the IRIS and the CML-IV study, suggesting that a TKI dose reduction of at least 50% does not affect long-term treatment. In addition, we found that the duration of dose reduction also did not affect the acquisition of TFR. Interestingly, Iurlo et al. (2022) found TFR was significantly better after dose reduction due to adverse events than those with dose de-escalation after DMR achievement. Consistent with the literature, our data showed that dose reductions do not hinder the achievement of TFR, and TFR might be independent of the degree of reduction of TKI.

In the univariate analysis, the type of TKI, TKI resistance, line of therapy, and time to MR4, was the clinical variable significantly associated with molecular recurrence. Multivariate analysis showed patients with TKI resistance presented with significantly lower TFR rates than those without resistance. Similarly, imatinib resistance is an important risk factor for molecular relapse in the DADI trial (Okada et al., 2018). In ENESTnd 10-year analysis, patients treated with first-line nilotinib achieved a higher rate of TFR eligibility compared to first-line imatinib (Kantarjian et al., 2021). A previous study also showed that first-line 2-3G TKIs compared to imatinib were significantly associated with a better TFR (Etienne et al., 2020). In the present study, we compared the TFR of patients treated with imatinib continuously and patients transferred to 2G-TKI from imatinib. We found patients transferred to 2G-TKI treatment for imatinib resistance or non-resistance reasons had remarkably higher TFR rates than those who did not. In addition, patients who switched to 2G-TKI therapy for imatinib resistance or non-resistance reasons achieved similar TFR rates compared to patients treated with 2G-TKI in the first line. Likewise, patients treated with nilotinib as first-line and second-line experienced a similar TFR rate (58% vs. 51.6%) at 48 weeks in the ENESTfreedom study and the ENESTop study (Mahon et al., 2018; Radich et al., 2021). A recent study showed that patients treated with 2G-TKI as

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TABLE 3 Univariate and multivariable analysis of various parameters' associations with molecular relapse.

Characteristic	Probability of remaining MMR at 2 years	*p* (Log-rank)	Multivariate analysis HR (95% CI)	*p*
Age, years		0.442		
≤43	68.1 (55.7–77.7)			
>43	62.9 (51.2–72.5)			
Sex (		0.056		
Male	58.5 (46.4–68.7)			
female	73.0 (61.9–81.3)			
Previous IFN-α treatment		0.578		
Yes	52.4 (16.2–79.5)			
No	66.8 (58.6–73.7)			
TKI at discontinuation		0.001		
Imatinib (ref.)	54.3 (43.0–64.3)		0.585 (0.140–2.436)	0.461
2-G TKI	79.3 (68.1–86.9)			
TKI resistance		0.015		
Yes	50.0 (31.8–65.7)			
No (ref.)	70.2 (61.0–77.6)		3.892 (1.811–8.365)	<0.001
Line of therapy		0.003		
1st (ref.)	56.5 (45.7–66.0)		0.298 (0.062–1.443)	0.133
≥2nd	78.8 (66.8–86.8)			
Sokal		0.791		
Low	72.5 (58.0–82.8)			
Intermediate	60.8 (33.8–79.6)			
High	56.0 (24.9–78.5)			
ELTS		0.672		
Low	73.3 (60.1–82.7)			
Intermediate	54.6 (30.0–73.7)			
High	67.5 (29.1–88.2)			
Duration of TKI, mo		0.736		
≤82	61.8 (49.8–71.7)			
>82	70.7 (59.8–79.2)			
Time to MR4, mo		<0.001		
≤12 (ref.)	78.6 (69.0–85.5)		1.611 (0.856–3.032)	0.140
>12	46.4 (32.0–59.6)			
Duration of MR4, mo		0.081		
≤61	57.0 (44.8–67.4)			
>61	75.3 (64.9–83.1)			
Low-dose of TKI		0.320		
Yes	55.8 (30.3–75.2)			
No	66.7 (58.0–74.0)			

2-G TKI, second-generation tyrosine kinase inhibitor.

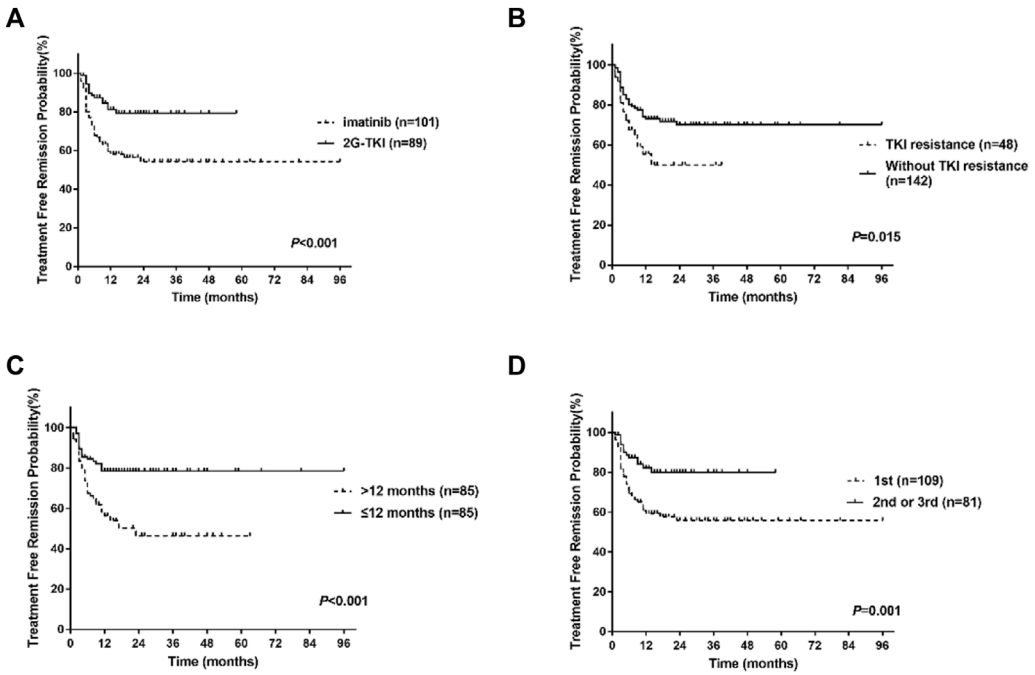

FIGURE 3
Treatment-free remission (TFR) after tyrosine kinase inhibitor (TKI) therapy. **(A)** TFR in patients according to the type of TKI at discontinuation. **(B)** TFR in patients according to TKI resistance. **(C)** TFR in patients according to the time to MR4. **(D)** TFR in patients according to the line of TKI. 2G-TKI, second-generation TKI.

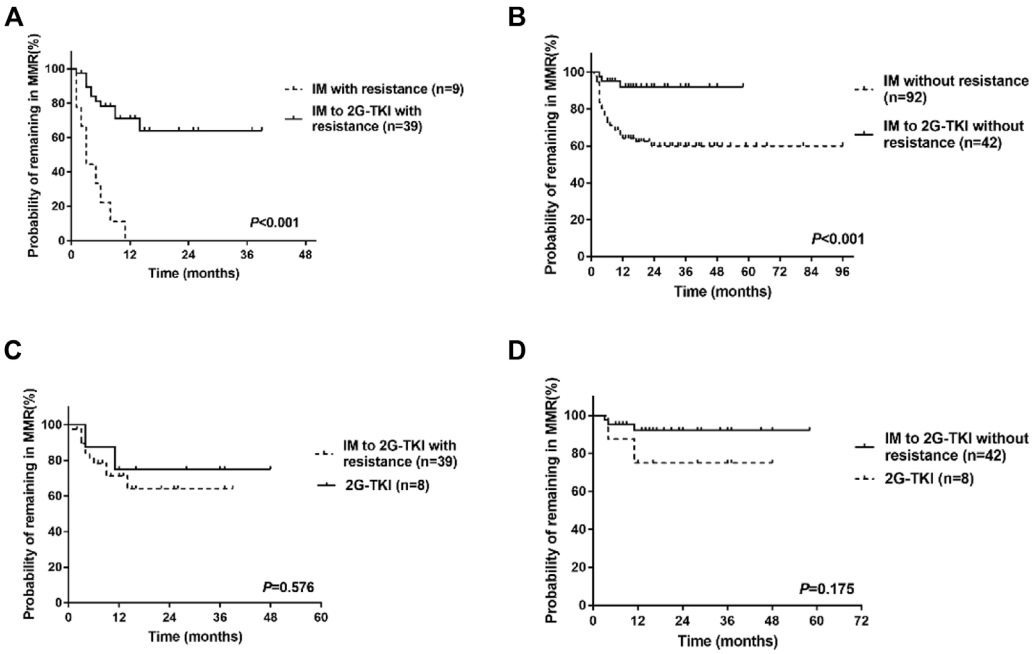

FIGURE 4
Treatment-free remission (TFR) in patients after tyrosine kinase inhibitor (TKI) therapy. **(A)** TFR in patients with imatinib resistance. **(B)** TFR in patients without resistance. **(C)** TFR in patients who switched to 2G-TKI for imatinib resistance or patients with 2G-TKI. **(D)** TFR in patients who switched to 2G-TKI therapy without imatinib resistance or patients with 2G-TKI. IM, imatinib; 2G-TKI = second-generation TKI.

first-line treatment and at discontinuation had a lower net probability of TKI re-initiation (p = 0·017) compared with patients treated with imatinib (Flygt et al., 2021). In general, our results combined with data from other studies suggested that for patients with a need for discontinuation, second-generation TKI therapy may yield higher TFR rates than imatinib, especially for imatinib-resistant patients.

Several studies reported some patients developed or worsened musculoskeletal pain after discontinuing TKI, suggesting the presence of withdrawal syndrome. For example, the KID study reported that 30% of patients underwent withdrawal syndrome after TKI withdrawal (Lee et al., 2016). Berger et al. (2019) analyzed the withdrawal syndrome after stopping TKIs by combining the patients included in the STIM2 and EURO-SKI trials and showed that 23.2% of patients developed withdrawal syndrome. In addition, 21% of patients in the DESTINY study reported new musculoskeletal symptoms during the dose-reduction phase (Clark et al., 2017). In the present study, 14 patients (7.4%) developed transient musculoskeletal pain within 12 months after TKI discontinuation. Thirteen patients reported a grade 1–2 TKI withdrawal syndrome and one patient reported a grade 3 TKI withdrawal syndrome. Three patients lost MMR after the occurrence of musculoskeletal pain.

5 Conclusion

In conclusion, this retrospective study showed TKI discontinuation in the real-world is as feasible and safe as in clinical studies. Indeed, dose optimization is an important consideration in individualized treatment for patients with different demands. Moreover, we found TFR rates were not impaired in patients experiencing dose reduction before TKI discontinuation compared to patients with full-dose TKI. Our data may provide a basis for the safety and feasibility of dose optimization before drug withdrawal. For patients with a need for discontinuation, second-generation TKI therapy may yield higher TFR rates than imatinib, especially for imatinib-resistant patients. However, our study still has limitations as a retrospective study. In addition, only 27 patients discontinued low-dose TKI in this study, and the data on discontinuation after dose reduction may be biased. Some patients with a long history of disease did not have BCR-ABL1 mutations test, so we did not analyze the effect of BCR-ABL1 mutations on TFR. As real-world data are still increasing, more studies are needed to accumulate data in daily practice to confirm the feasibility of dose reduction.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by The Institutional Review Board of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

YC, HZ, WL, and YZ designed research, collected data, and wrote the manuscript. YC, HZ interpreted the data and performed statistical analysis. YC, WL, and YZ designed and supervised the overall study. JG, JZ, WH, DH, and FC collected data and reviewed the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Long-term outcomes of third-line therapy with tyrosine kinase inhibitors in chronic phase chronic myeloid leukemia: A real-life experience

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Introduction: Tyrosine kinase inhibitor (TKI) therapy has greatly improved the prognosis of patients with chronic myeloid leukemia (CML), improving the survival expectancy of patients with chronic phase (CP) CML to that of the general population. However, despite these advances, nearly 50% of patients with CP CML experience failure to respond to frontline therapy, and most fail to respond to the subsequent second-line TKI. Treatment guidelines for patients failing second-line therapy are lacking. This study aimed to determine the efficacy of TKIs as third-line therapy in a "real-world" clinical practice setting and identify factors favorably influencing the long-term outcomes of therapy.

Methods: We have retrospectively analyzed the medical records of 100 patients with CP CML.

Results: The median age of the patients was 51 (range, 21–88) years, and 36% of the patients were men. The median duration of the third-line TKI therapy was 22 (range, 1– 147) months. Overall, the rate of achieving complete cytogenetic response (CCyR) was 35%. Among the four patient groups with different levels of responses at baseline, the best results were achieved in the groups with any CyR at the baseline of third-line therapy. Thus, CCyR was reached in all 15 and 8/ 16 (50%) patients with partial cytogenetic response (PCyR) or minimal or minor CyR (mmCyR), respectively, whereas CCyR was detected only in 12/69 (17%) patients without any CyR at baseline ($p < 0.001$). Univariate regression analysis revealed that the factors negatively associated with CCyR achievement in thirdline TKI therapy were the absence of any CyR on first- or second-line TKI therapy ($p < 0.001$), absence of CHR prior to third-line TKI ($p = 0.003$), and absence of any CyR prior to third-line TKI ($p < 0.001$). During the median observation time from treatment initiation to the last visit [56 (4–180) months], 27% of cases progressed into accelerated phase or blast phase CML, and 32% of patients died.

Discussion: Progression-free survival (PFS) and overall survival (OS) were significantly higher in patients with CCyR on third-line than in the group without CCyR on third-line therapy. At the last visit, third-line TKI therapy was ongoing in 18% of patients, with a median time of treatment exposure of 58 (range, 6–140) months; 83% of these patients had stable and durable CCyR, suggesting that patients without CHR at baseline and without CCyR at least by 12 months on third-line TKI should be candidates for allogeneic stem cell transplantation, third-generation TKIs, or experimental therapies.

KEYWORDS

chronic myeloid leukemia, chronic phase, efficacy of therapy, complete cytogenetic response, third-line therapy

1 Introduction

Tyrosine kinase inhibitors (TKIs) have significantly improved the outcomes of patients with chronic myeloid leukemia (CML), seemingly increasing their life expectancy to that of the general population (1). However, 35%–45% of patients fail to respond to first-line TKI within 10 years of therapy (2, 3). Most patients after first-line treatment discontinuation are switched to second-generation TKI. Overall, nilotinib, dasatinib, and bosutinib have demonstrated similar response rates, with probabilities of obtaining a complete cytogenetic response (CCyR) of approximately 50%. After 5 years of observation, up to 60% of patients discontinue second-line therapy mainly due to primary or secondary resistance (4–6). Treatment and response recommendations focus on first- and second-line therapy with first-generation (imatinib) or second-generation (nilotinib, dasatinib, or bosutinib) TKIs (7, 8). Currently, the ELN 2020 guidelines suggest the use of the third-generation TKI ponatinib as third-line therapy in patients without specific mutations rather than alternative second-generation TKIs. Patients with suboptimal response to two or more previous TKIs should be transferred to allogeneic stem cell transplantation (allo-SCT) (7). Allo-SCT may be considered for patients with *de-novo* blast phase (BP) CML, preferably after achieving some response to a TKI-based therapy, those with accelerated phase (AP) CML who are not responding well to current therapy, those who progressed to AP/BP CML while receiving TKI therapy, and those with resistance or intolerance to TKIs. It may also be used in patients with T315I mutation after an inadequate response to attempted ponatinib therapy (9). However, the possibilities for its implementation are limited due to the risks associated with age, comorbidities, and/or lack of matched donors. Globally, only a small proportion (≤1%) of patients with chronic phase (CP) CML undergo allo-SCT after failure of second-line therapy, while most patients continue to receive other TKIs (10).

It is very important to identify a group of patients in whom third-line therapy with TKIs is associated with equal or even better long-term outcomes than using allo-SCT. As a reference point for determining the effectiveness of third-line TKI, an assessment of the achievement of CCyR has been proposed. CyR, especially CCyR, has historically been and is currently associated with a significant survival advantage in patients with CP CML (11, 12).

This study aimed to evaluate the long-term efficacy of third-line therapy with available TKIs and identify the factors that could favorably influence CCyR achievement and survival prognosis.

2 Materials and methods

2.1 Patients

This multicenter retrospective observational study that aimed to evaluate the long-term efficacy of third-line therapy with TKI (third TKI) in patients with CP CML in real-life practice was conducted between 2019 and 2022. The inclusion criteria were as follows: 1) age ≥18 years, 2) CP CML (ELN criteria 2013), 3) treatment with available third-line TKI outside clinical trials with any reason of previous discontinuation, and 4) absence of CCyR at baseline. The exclusion criteria were 1) history of AP/BP and 2) prior allo-HSCT. All patients gave informed consent prior to participation. All study procedures were performed in accordance with the institutional and national ethical standards on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. Overall, 100 [men, n = 36 (36%)] patients from six centers of the Saint Petersburg and Leningrad region were included. The data of 73 patients were collected primarily in 2019 and updated in 2022. The data of the other 27 patients were collected in 2022. In all cases, the diagnosis of CML was confirmed using cytogenetic and molecular analysis (13). At the time of diagnosis and time of initiation of third-line TKI, the median age of the patients was 45 (range, 12–82) and 51 (range, 21–88) years, respectively. First-line TKI treatment for most patients was imatinib (n = 97, 97%). Nilotinib, dasatinib, imatinib, and bosutinib were used as second-line therapy in 69 (69%), 25 (25%), 1 (1%), and 5 (5%) patients, respectively. The median duration of CML from the time of diagnosis to first-line TKI and third-line TKI and from the initiation of first-line TKI and second-line TKI therapy to the initiation of third-line therapy was 2 (range, 1–245), 64 (range, 7–316), 48 (range, 7–156), and 17 (3–96) months, respectively. The main characteristics of the patients prior to third-line TKI initiation are shown in Table 1.

Overall, any time before the initiation of third-line TKI therapy, *BCR::ABL* mutational analysis was performed in 91/100 (91%) patients. *BCR::ABL* mutations were evaluated on both first- and second-line

TABLE 1 Characteristics of patients on first- and second-line TKI therapy (n = 100).

Patient characteristics		First-line TKI	Second-line TKI	On first- and second-line TKI
Median duration (range)		22 (2–145)	14 (0.5–96)	46 (6–156)
Best responses, n (%)	No CHR	17 (17%)	13 (13%)	3 (3%)
	CHR without CyR	35 (35%)	36 (36%)	32 (32%)
	mmCyR	16 (16%)	17 (17%)	13 (13%)
	PCyR	9 (9%)	12 (12%)	17 (17%)
	CCyR	17 (17%)	14 (16%)	22 (22%)
	MMR	6 (6%)	8 (8%)	13 (13%)
	MR ≥4	0 (0%)	0 (0%)	0 (0%)
Time to best response, median (range), months		6 (1–67)	5 (1–24)	6 (1–61.5)
Reason for TKI withdrawal, n (%)	Resistance	90 (90%)	72 (72%)	72%
	Intolerance	10 (10%)	28 (28%)	24% continued therapy; 4% discontinued both TKIs due to resistance and intolerance
BCR::ABL mutations	Detected in evaluable patients any time on TKI, n (%)	10/33 (30%)	32/66 (48%)	40/91 (44%)
	Type of mutations	G250E—3 E255K—1 E255V—2 M351T—1 D363Y—1 H396P—1 Q252H +E255K—1	L248V—1 E355A—1 G250E—5 E255V—1 Y253F—1 Q252H—1 Y253H—6 F311C—2 T315I—6 F317L–4 F359C–1 T315I+Y253H —1 F317L+F359V —1 F317L+L248V —1	G250E—8 E255V—3 M351T—1 D363Y—1 H396P—1 F359C+Q252H+E255K—1 E255K+L248V—1 E355A—1 Y253F—1 Q252H—1 Y253H—6 F311C—2 T315I—6 F317L—4 T315I+Y253H—1 F317L+F359V—1 F317L+L248V—1

TKI, tyrosine kinase inhibitor; CHR, complete hematologic response; CyR, cytogenetic response; mmCyR, minimal or minor cytogenetic response; PCyR, partial cytogenetic response; CCyR, complete cytogenetic response; MMR, major molecular response; MR ≥4, deep molecular response ≥4 log.

TKIs only in 8/91 (8%) cases. Mutations were identified in 40/91 (43.9%) evaluable cases. After failure to respond to first-line TKI, 10 mutations were identified in 33 (30%) patients, with one patient having two mutations. Only five clinically significant mutations (G250E, E255K, E255V, M351T, and Q252H) in seven patients were detected. After failure to respond to second-line therapy, TKI *BCR::ABL* kinase domain point mutations were identified in 32/66 patients (48%), with mutations being repeatedly identified in two patients following first-line therapy. Only eight clinically significant mutations in 27 patients (G250E, E255V, Q252H, T315I, Y253H, F317L, F359C, and L248V) were detected. The T315I mutation was detected in 7/91 (8%) patients.

Additional chromosomal aberrations were detected in 19/100 (19%) patients at any time prior to third-line therapy.

At the baseline of third-line TKI therapy, 35 (35%), 34 (34%), 16 (16%), and 15 (15%) patients did not have a complete hematologic response (CHR) and had CHR without cytogenetic response (CyR), minor or minimal CyR (mmCyR), and partial CyR (PCyR), respectively. No patients with CCyR or deeper responses at baseline were included in the study according to the inclusion criteria.

2.2 Statistical analysis

The cumulative rate of CCyR achievement on third-line TKI was assessed as the primary efficacy endpoint of third-line TKI. All decimal numbers were converted to the nearest whole number. CCyR was defined as 0% of Ph+ metaphases, with at least 20 metaphases required, according to bone marrow cytogenetics or 2-log reduction of molecular response (MR2), with a *BCR::ABL/ABL* ratio of ≤1% in peripheral blood by reverse transcription quantitative real-time polymerase chain reaction test (14).

To test the type of distribution of quantitative variables, the Shapiro–Wilk *W*-test was used. Variables did not have a normal distribution in any of the cases. Continuous variables were reported as medians and ranges. Categorical variables were reported as absolute values and percentages. Differences between categorical variables were calculated using the χ^2 test, and differences between continuous variables were calculated using the Mann–Whitney *U* test.

Overall survival (OS) was defined as the time from the initiation of third-line TKI therapy to death. Progression-free survival (PFS) was defined as the time from the initiation of third-line TKI therapy to disease progression or death, whichever occurred first. Event-free survival (EFS) was defined as the time from the initiation of third-line therapy to therapy withdrawal, disease progression, or death, whichever occured first (15). OS and PFS were calculated using the Kaplan–Meier method. The log-rank test was used to assess the significance of differences in survival between the patient groups.

The influence of possible risk factors on CCyR achievement was assessed by univariate analysis.

3 Results

At the time of the last data collection, the median duration of third-line TKI therapy was 22 (range, 1–147) months, and the median time of follow-up from the initiation of third-line TKI and from diagnosis to the last visit was 56 (range, 4–180) months and 125 (range, 27–434) months, respectively. Only four patients were lost to follow-up after a median observation time of 29 (range, 5–79) months.

Patients received dasatinib, nilotinib, bosutinib, or ponatinib as third-line therapy in 58 (58%), 22 (22%), 17 (17%), and 3 (3%) cases, respectively.

3.1 Response to therapy

3.1.1 CHR

At baseline, 65 patients had CHR, and 26/35 (74%) patients who did not have CHR at baseline achieved it by 3 months of therapy. CHR was subsequently lost in 9/26 (35%) and 5/65 (8%) patients without and with CHR at baseline, respectively ($p = 0.002$).

3.1.2 CCyR

With a median time on third-line TKI therapy of 22 (range, 1–147) months, CCyR (or MR2) was achieved in 35/100 (35%) patients. The median time to response was 6 (range, 2–22) months. Most patients (23/35; 66%) obtained CCyR within 6 months of treatment. No patients with prolonged treatment of third-line TKI reached CCyR after 24 months.

The influence of the baseline level of response to CCyR in patients undergoing third-line TKI therapy was assessed. The median time on third-line TKI therapy for patients without CHR, with CHR but no CyR, and with any CyR (mmCyR or PCyR) was 20 (range, 1–95), 18 (range, 1–147), 34 (range, 5–140), and 32.5 (range, 3.5–121) months, respectively. CCyR was reached in 30/65 (46%) and 5/35 (14%) patients with and without CHR at baseline ($p = 0.02$). No differences in CCyR achievement were observed in patients without CHR and with CHR but no CyR ($p > 0.05$). Meanwhile, CCyR was significantly higher in patients with any baseline CyR than in patients with no CyR with or without CHR ($p < 0.05$). Moreover, all patients with any CyR at baseline reached CCyR during therapy ($p = 0.013$). The rate of CCyR in the patient groups is presented in Table 2.

CCyR was also higher in patients without clinically significant *BCR::ABL* mutations than in those with such mutations. Thus, CCyR was obtained in 8/34 (24%) and 27/57 (47%) evaluable patients with and without clinically significant mutations of *BCR::ABL*, respectively ($p = 0.024$). The probability of CCyR achievement by 12 and 24 months was 41% and 54%, respectively (Figure 1).

The expected CCyR by 12 months according to initial response was 12%, 31%, 52%, and 94% in patients without CHR, with CHR but no CyR, with mmCyR, and with PCyR, respectively (Figure 2).

Loss of CCyR was observed in 13/35 (37%) patients after a median time of 22 (range, 2–46) months. By the last visit, CCyR was sustained in 2/5 (40%), 4/7 (57%), 5/8 (62,5%), and 11/15 (73%) patients with no CHR, with CHR but no CyR, and with any CyR (mmCyR or PCyR), respectively. No statistical differences were observed among patients with different baseline levels of response. In patients with sustained CCyR, the median duration of response was much longer and reached 49 months (range, 2–144.2; $p < 0.05$).

TABLE 2 The cumulative rate of CCyR in patients undergoing third-line TKI therapy.

Cumulative rate of CCyR, *n* (%)	All patients (*n* = 100)	Baseline response			
		No CHR *n* = 35	CHR but no any CyR *n* = 34	mmCyR *n* = 16	PCyR *n* = 15
Any time	35 (35%)	5 (14%)	7 (20.5%)	8 (50%)	15 (100%)
By 6 months	23 (23%)	3 (9%)	5 (15%)	5 (31%)	10 (67%)
By 12 months	30 (30%)	3 (9%)	7 (21%)	6 (37.5%)	14 (93%)

Time to CCyR achievement was shorter in patients with stable response than with loss of CCyR [4 (range, 2–22) *vs.* 8 (range, 2.5–15) months, respectively; $p > 0.05$], though this difference was not statistically significant. The rate of CCyR loss was nearly similar in patients that achieved CCyR by 6 months of therapy (6/23; 26%) and those that achieved it later (7/12, 58%; $p = 0.06$).

Among patients with CCyR, 17/35 (49%) obtained a major molecular response (MMR) or better molecular responses. At the last visit, all patients except three had stable MMR or better molecular response. CCyR was lost only in 1/17 (6%) and 12/18 (67%) patients with and without MMR, respectively ($p = 0.001$).

3.2 Factors influencing the probability of CCyR achievement

Results of the univariate regression analysis (Table 3) revealed that the factors negatively associated with CCyR achievement during third-line TKI were as follows:

- Absence of any CyR during first- or second-line TKI therapy ($p < 0.001$)
- Absence of CHR prior to third-line TKI therapy ($p = 0.003$)
- Absence of any CyR prior to third-line TKI therapy ($p < 0.001$)

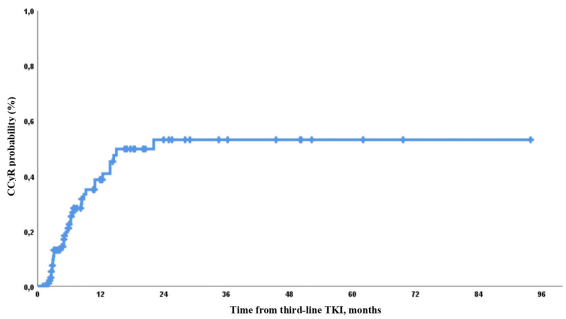

FIGURE 1
Probability of complete cytogenetic response (CCyR) achievement during third-line tyrosine kinase inhibitor (TKI) therapy.

3.3 Progression to advanced phases and PFS

During the median observation time of 56 (range, 3.5–179.5) months from treatment initiation to the last visit, 27/100 (27%) patients progressed into AP/BP CML. During third-line TKI treatment, AP/BP was registered in 10/27 (37%) patients, with a median time to progression of 12 (range, 4–39) months. In 17/27 (63%) patients with AP/BP after third-line TKI discontinuation, the median time to progression was 28 (range, 9–110) months. More than a quarter (7/27; 26%) of the progressions occurred within the first year of third-line TKI therapy. As for other cases, 4/27 (15%), 7/27 (26%), 5/27 (19%), and 4/27 (15%) were diagnosed with AP/BP CML during the second year, third year, between 3 and 5 years, and beyond 5 years of observation, respectively.

The median observation time of patients without CHR, with CHR but no CyR, and with any CyR (mmCyR or PCyR) was 44 (range, 5–169), 58 (range, 8–180), 63 (range, 5–140), and 56 (range, 4–121) months, respectively. As expected, most cases of AP/BP CML developed in patients without CHR at baseline ($n = 17/35$; 48.5%). A comparable proportion of patients without CHR at baseline progressed regardless of CHR achievement on third-line TKI therapy [achievement, 5/9 (56%); non-achievement, 12/26 (46%); $p = 0.6$]. The transformation rate was lower in the CHR but no CyR group (8/34; 23.5%) than in the no CHR group [17/35 (48.5%); $p = 0.031$]. Patients with any CyR at baseline had similar rates of progressions. Thus, AP and BP were diagnosed in 2/16 (12.5%) and 0/15 (0%) patients with any CyR, respectively ($p = 0.191$).

Thirty-five events were included in the analysis of PFS: 27/100 (27%) patients progressed, and 8 deaths occurred owing to the following reasons: transplant-related causes ($n = 3$) and other reasons ($n = 5$), including cardiovascular disease ($n = 3$), other secondary neoplastic processes ($n = 1$), and SARS-CoV-2 infection (1). The 1-, 2-, 3-, and 5-year PFS rates were 92%, 83%, 77%, and 68%, respectively (Figure 3).

The expected PFS was similar and was very high among patients with any CyR at baseline. It was lower in patients with CHR but no CyR than in other patients. There were no significant differences between patients with different levels of cytogenetic resistance at baseline. The worst PFS was observed in patients with hematologic resistance (Figure 4).

PFS events occurred in 2 and 33 patients with or without CCyR on third-line TKI, respectively. The 1-, 2-, 3-, and 5-year PFS rates were 97%, 97%, 97%, and 93% in patients with CCyR achievement

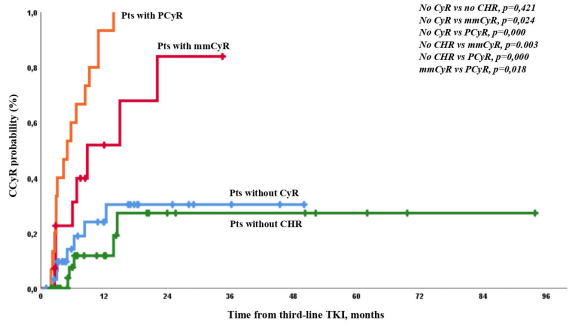

FIGURE 2
Probability of CCyR achievement in the different groups of patients according to the level of response at baseline.

on treatment and 88%, 72%, 63%, and 52% in patients with no CCyR on treatment, respectively, with significant differences at any time point (Figure 5).

3.4 OS

With the median observation time from the beginning of third-line therapy to the last visit of 56 (range, 3.5–179.5) months, 32/100 (32%) deaths occurred, 21 of which were due to CML progression, 6 occurred after complications of allo-SCT (n = 3, allo-SCT was performed due to BP), and 5 occurred for other reasons [cardiovascular disease, n = 3; other secondary neoplastic processes, n = 1; and SARS-CoV-2 infection, n = 1 (1)]. Drug-related toxicity did not cause any deaths (Table 4). The causes of death in different groups are shown in Table 4.

Deaths while on third-line TKI were observed in 5/32 (16%) cases, with a median time to death of 30 (range, 4–50) months. After third-line therapy discontinuation, there were 27/32 (63%) cases of death, with a median time to mortality of 25 (range, 7–114) months. Deaths were observed in 6/100 (6%), 14/100 (14%), 20/100 (20%), and 28 (28%) cases by 12, 24, 36, and 60 months from third-line TKI, respectively. OS was 92%, 83%, 77%, and 68% by 12, 24, 36, and 60 months of observation, respectively (Figure 6).

After a nearly equal observation time from third-line treatment, it was found that the rate of death was lower in the group with any depth of CyR than in the group with no CyR but with CHR and the group without CHR at baseline [2/31 (6.5%) *vs.* 10/34 (29%) *vs.* 20/35 (57%), $p < 0.01$]. Simultaneously, patients with mmCyR and PCyR have a similar mortality rate of 1/16 (6%) *vs.* 1/15 (7%) (p = 0.9), respectively.

The expected OS was the lowest in patients with no initial CHR. It was higher in patients with any CyR than in patients with CHR but no CyR (Figure 7).

No differences in mortality rate between patients without CHR at baseline but who reached and did not reach CHR during third-line therapy were observed [14/26 (54%) and 6/9 (67%), respectively, p = 0.5]. Meanwhile, there were only 2/35 (6%) and 30/65 (46%) deaths among patients who achieved and did not achieve CCyR at any time on third-line TKI (p = 0.0001). OS was 97% *vs.* 90%, 90% *vs.* 77%, 90% *vs.* 67%, and 93% *vs.* 55% by 12, 24, 36, and 60 months of observation, respectively, between patients with CCyR who achieved or did not achieve CCyR during therapy (Figure 8).

3.5 EFS and patients' current status

With a median time of third-line TKI of 22 (range, 1.0–147) months, third-line TKI was discontinued in 82/100 (82%) patients in the whole group. TKI withdrawal due to resistance, death unrelated to CML, TKI toxicity, and treatment-free remission occurred in 66/82 (80%), 5/82 (6%), 10/82 (12%), and 1/82 (1%) cases, respectively.

EFS was defined as the time from the initiation of third-line therapy to therapy withdrawal or to disease progression or death, whichever occured first, as described earlier. Therapy discontinuation during deep molecular response for treatment-free remission was censored at the time of drug withdrawal.

TABLE 3 Univariate regression analysis for the risk of not achieving a CCyR on third-line TKI therapy.

Prognostic factor	OR	95% CI	*p*-value
Sex, male	1.36	0.58-3.34	0.485
Age at start of third-line TKI (per 10 years)	1.1	0.83-1.48	0.514
Age at start of third-line TKI >60 years	1.19	0.48-3.12	0.709
Time from diagnosis to third-line TKI (per year)	0.997	0.99-1.004	0.395
Time from first- to third-line TKI (per year)	0.999	0.987-1.011	0.873
Absence of CyR on first-line or second-line TKI	0.063	0.01-0.23	<0.001*
Absence of CHR prior to third-line TKI	0.194	0.06-0.53	0.003*
Absence of CyR prior to third-line TKI	0.073	0.025-0.19	<0.001*
Mutations on second-line TKI	1.17	0.38-3.63	0.777

OR, odds ratio.
*Indicates statistical significance ($p < 0.05$).

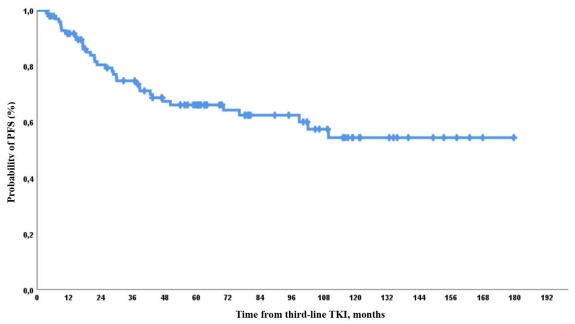

FIGURE 3
Progression-free survival (PFS) on third-line TKI therapy.

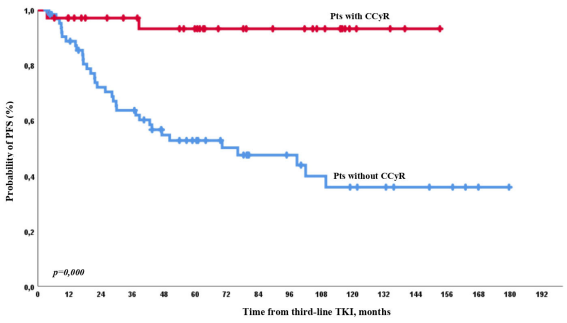

FIGURE 5
PFS on third-line therapy according to CCyR achievement on third-line TKI.

There were 67/100 (67%) withdrawals, 10/100 (10%) progressions, and 5/100 (5%) deaths. Thus, 18/100 (18%) patients continued third-line TKI therapy.

EFS by 12, 24, 36, and 60 months was 65%, 48%, 37%, and 24%, respectively (Figure 9).

There were 35/100 (35%) with CCyR achievement on third-line TKI, 20/35 (57%) of whom discontinued third-line therapy. The reasons for discontinuation were as follows: progression in 1/20 (5%), death because of other reasons (cardiovascular disease complications) in 1/20 (5%), treatment-free remission in 1/20 (5%), secondary molecular resistance in 1/20 (5%), secondary cytogenetic resistance in 13/20 (65%), and toxicity in 3/20 (15%). At the last visit among patients with CCyR, 9/15 (60%) patients had an MMR or better molecular response. The long-term outcomes are presented in Figure 10.

4 Discussion

Most patients were treated with dasatinib and nilotinib as third-line TKI. Bosutinib was the treatment of choice only in 17% of patients. The drug was registered in our country much later than other TKIs and was available for fewer patients. Ponatinib was not registered in the country at the time of data collection. Only three patients were provided with ponatinib *via* different programs outside of clinical trials. Owing to recent advances in TKI therapy, patients with CP CML have a low incidence of progression to AP/BP and CML-related death. Moreover, up to 30% of patients with a stable deep molecular response have a chance of treatment-free remission. Meanwhile, up to 45% and 60% of patients, respectively, discontinue first- and second-line TKI therapy due to resistance or intolerance. Current guidelines consider ponatinib and allo-SCT as a third-line strategy, especially for patients with TKI resistance, but in real-life practice, nearly all patients are switched to third-line TKI therapy with second-generation TKI because of different socioeconomic status and inaccessibility of third-generation TKI. In our previous study (16), only 21% of new cases of CCyR among patients with CP CML undergoing second-generation TKI therapy continued to third-line therapy, and 21/53 (40%) patients experienced drug withdrawal in less than 2 years of observation. The 2-year OS was 67%. All patients with major CyR on third-line TKI were in stable CP and alive during observation (16). In our current retrospective study, CCyR was a significant marker for both PFR and OS. Among the 35 patients with CCyR, only one patient died due to progression to AP/BP and another one died for a reason not related to CML. Both PFS and OS were significantly higher at any time point in patients with CCyR than in patients with no CCyR during third-line treatment. In these groups of patients, the 5-year PFS and OS were 93% *vs.* 52% and 93% *vs.* 55%, respectively ($p < 0.05$). According to the results of our study, 27/100 (27%) patients progressed to advanced phases. Progression to BP occurred mostly among patients without CHR at baseline [n = 17/35 (49%)]. There were only two progressions in the mmCyR group [2/16 (12.5%)]. Disease progression did not occur among patients with PCyR at baseline. Similar results were shown by Garg et al. (17), where transformation to AP/BP was reported in 12/48 (25%) patients. Among patients with AP/BP progression, most [8/12 (67%)] did not achieve any CyR prior to third-line TKI therapy (17). In an article by Ribeiro et al. (18), among 18 patients with CP CML, only 3/18 (16%) progressed to BP on third-line therapy. Khan et al. (19) reported that the depth of response to therapy (both CCyR and MMR) was associated with improved OS in the univariate analysis. In a

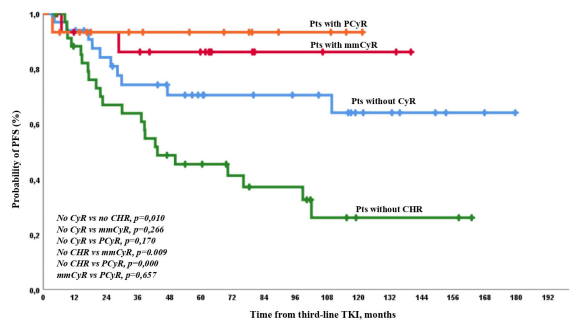

FIGURE 4
PFS on third-line TKI therapy according to the depth of response at baseline. There were only 1/35 (3%) and 26/65 (40%) cases of AP/BP among patients who achieved or did not achieve CCyR on third-line TKI therapy (p = 0.0001).

TABLE 4 Causes of death according to the depth of response at baseline.

Cause of death	Patients without CHR	Patients without CyR	Patients with mmCyR	Patients with PCyR	All	*p*
Progression	13	8		0	21 (65%)	<0.01
Complications after allo-SCT	5	0	1		6 (19%)	
Deaths from other causes	2	2	0	1	5 (16%)	
All	20 (63%)	10 (31%)	1 (3%)	1 (3%)	32 (100%)	

multivariate analysis, MMR was a favorable prognostic marker for OS (19). In our study group, MMR or better molecular responses were reached in 17/35 (49%) patients with CCyR. All patients were alive at the last visit, and 14/17 (82%) patients had stable MMR or better molecular response. CCyR loss was observed only in one out of 17 (6%) cases among patients with MMR achievement, as compared with 12/18 (67%) cases of CCyR without MMR achievement (p = 0.001). As only two patients died in the CCyR group, no difference was considered present between patients with and without MMR achievement during observation. In our observation, 35/100 (35%) patients reached CCyR. In our study, loss of CCyR on third-line therapy occurred in 13/35 (37%) patients. Ongoren et al. (20) reported that most patients on third-line TKI therapy achieved CCyR [11/21 (52%)], and only one patient lost CCyR on third-line therapy [1/11 (9%)] (20).

While CCyR was a strong surrogate marker of survival on third-line TKI, we have attempted to search for factors that influenced CCyR achievement. The baseline level of response and also prior responses and mutations were studied as prognostic factors for CCyR achievement. Among the four patient groups with different responses at baseline, the best results were achieved in the groups with any CyR at baseline. Thus, CCyR was reached in 23/31 (74%) patients with any level of CyR compared with 12/69 (17%) patients without any CyR at baseline (p = 0.0001). These results were compatible with the outcomes of the study of Ibrahim et al. (21), which showed that the rate of the 30-month cumulative incidence of CCyR in subgroups with and without any CyR was 71% and 0%, respectively (p = 0.0005). The significance of the depth of cytogenetic response before third-line therapy was also assessed in the study of Bosi et al. (22), where among the 13 patients who failed to respond to first- and second-line therapy, six with cytogenetic response before third-line therapy achieved a deeper cytogenetic response and higher overall survival on third-line TKI therapy.

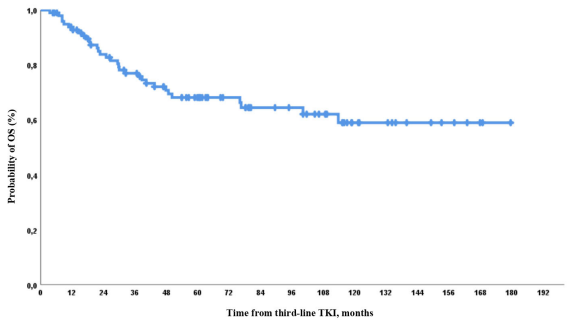

FIGURE 6
Overall survival (OS) on third-line therapy.

We performed univariate analyses to identify pretreatment factors that predicted complete cytogenetic response. In the univariate analysis, the factors found to have a significant influence on the probability of achieving a complete cytogenetic response were age at third-line therapy, CyR on first- or second-line therapy, CyR before third-line therapy, and CHR before third-line therapy. The presence of kinase domain mutations prior to third-line therapy did not affect the probability of achieving a complete cytogenetic response, as discussed previously. For the multiple regression analysis, we included also the time from the diagnosis to third-line therapy as a factor that reliably affects the achievement of a cytogenetic response according to the data of global clinical practice (23).

Of note, most patients with CCyR on third-line TKI reached it within the first 12 months of therapy. Thus, the cumulative incidence of CCyR was 66% and 86% by 6 and 12 months of treatment, respectively.

Prior cytogenetic response is the most robust positive prognostic factor identified in patients with CML receiving TKIs in third-line therapy. This fact has been confirmed by our research, in addition to other studies. Ibrahim et al. (21) reported that CCyR on first- or second-line therapy was the only factor that affected the outcomes of third-line therapy (21). Third- and fourth-line therapy with bosutinib was also successful in the subgroup with any cytogenetic response at baseline. CCyR was achieved in 94% (31/33) of patients with any CyR at baseline in contrast, and the probability to achieve CCyR in the group without any CyR at baseline was 25% (7/28) (24). Again, in the study of Russo Rossi et al. (25), patients who did achieve a CyR on imatinib or had low and intermediate Sokal risk had a higher probability of achieving CyR with third-line TKI therapy ($p < 0.001$).

Baseline factors also influenced PFS and OS. The 5-year OS on third-line therapy in our study was 68%. Ribeiro et al. (18) reported higher 5-year OS rates among patients with CP CML (86%) than among patients with AP/BP CML. In the study of Cortes et al. (26), the 4-year OS among patients treated with third-line bosutinib was 78%. We could speculate that some patients took a longer time to reach third-line TKI, had more clinically significant *BCR::ABL* gene mutations, especially with T315I, or had other prior unfavorable

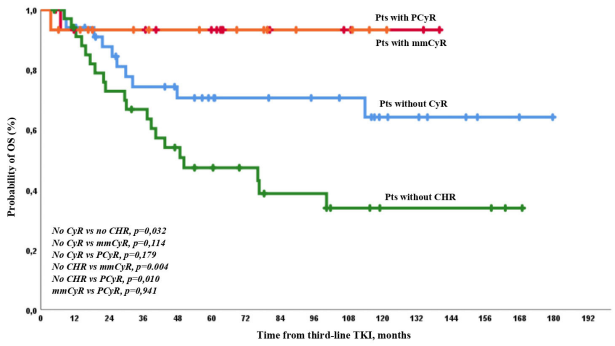

FIGURE 7
OS on third-line therapy according to the depth of response at baseline.

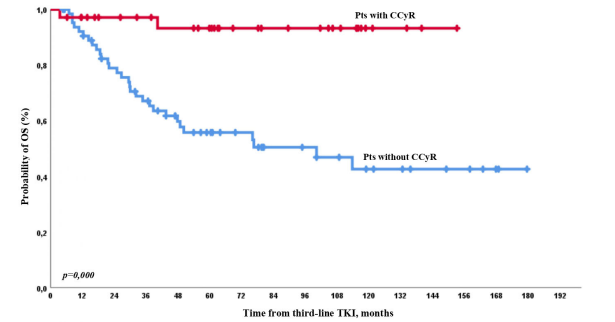

FIGURE 8
OS on third-line therapy according to CCyR achievement on third-line TKI.

factors compared with the patients in the aforementioned study, although there were not enough data for comparison. When comparing baseline factors, hematologic resistance was associated with the worst outcomes pertaining to PFS and OS, although we did

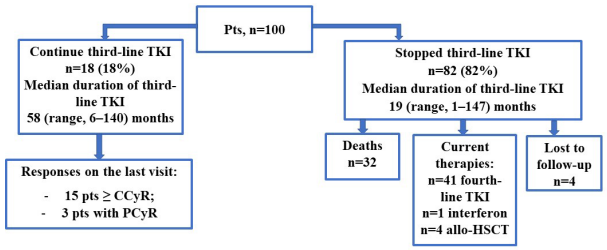

FIGURE 10
Long-term outcomes in the whole group on and after third-line TKI.

not find any statistical differences between groups with any level of cytogenetic responses at third-line TKI initiation. Most of our patients who failed to respond to therapy were switched to third-generation TKIs as their fourth or even fifth form of therapy within clinical trials; accordingly, we suspect that the high survival rate may be associated with subsequent therapies. Although we did not include these data in this analysis, many patients continued conservative TKI therapy without any CyR but were still in CP. We can speculate that patients with unfavorable biological changes, including genetic aberrations outside of *BCR::ABL*, progressed earlier while on first- or second-line TKIs and did not reach third-line treatment. Accordingly, a population-based study to evaluate the real unmet needs of patients on third-line therapy is warranted. Another obvious limitation of our study is its retrospective nature; accordingly, there were some gaps in data for some indicators and analysis. Moreover, the sample size was small; therefore, some of the analyses lacked statistical power to draw a clear conclusion. However, in our opinion, we have obtained interesting findings through the current retrospective research, which may allow us, along with the data of other authors, to define a therapeutic strategy in patients who responded to the first two generations of TKI therapy. In our observation, long-term treatment outcomes are not favorable as more than 80% of the patients discontinued third-line TKIs mainly due to resistance. These results can be improved by using ponatinib or asciminib.

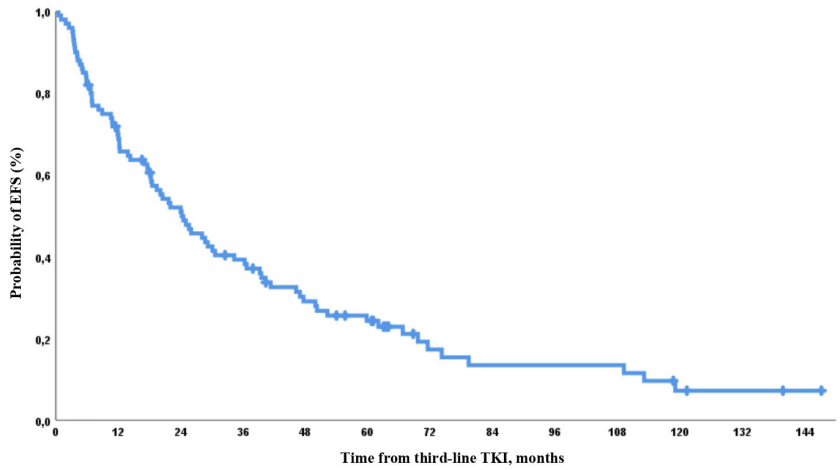

FIGURE 9
EFS on third-line therapy in the whole group.

5 Conclusion

TKI has revolutionized CML management, and the availability of different TKIs provides patients with some alternatives after failure to respond to first- and second-line therapy (27). The data reviewed here suggest that independent prognostic factors may be helpful for the identification of patients who are unlikely to achieve CCyR response on TKIs after failure to respond to imatinib or a prior second-generation TKI. The cytogenetic response prior to third-line therapy, as well as some degree of cytogenetic response during first- or second-line therapy, was proven to be highly informative. Patients with these two criteria had a much higher probability of achieving CCyR on third-line therapy. Patients with CCyR and those without baseline hematologic resistance had better PFS and OS than the remaining patients. Patients with any level of CyR response on previous TKIs, especially with PCyR, are seemingly good candidates for continued conservative therapy, even with second-generation TKIs. These patients should be closely monitored for CyR and molecular responses. Patients without CCyR by 6 months or especially 12 months on third-line TKI, as well as patients with unfavorable baseline prognostic factors, especially those without CHR, should be switched to other available therapies, including allo-SCT, third-generation TKIs, or experimental drugs.

Data availability statement

The datasets presented in this article are not readily available because all patient data is anonymized. Requests to access the datasets should be directed to chitanava_tv@almazovcentre.ru.

Author contributions

EL, VS, EMo, SV, and YV contributed to the conception and design of the study. TC organized the database. TC and IuM performed the statistical analysis. TC, IuM, VS, SV, IM, YV, EE, EM, AP, TM, RV, EK, NI, NM, ND, TS, NS, OK, ES, NL, JA, DM, EM, and EL provided research materials. TC wrote the first draft of the manuscript. EL, IuM, VS, SV, and EM wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

EL received fees for lecturing and expert opinion from Novartis, Pfizer, Bristol Myers Squibb, and Fusion Pharma. VS received fees for lecturing and expert opinion from Novartis, Pfizer, Amgen, and AbbVie. SV received fees for lecturing and expert opinion from Pfizer, Novartis, Janssen, AstraZeneca, Sanofi, Biocad, and Sotex. DM received fees for lecturing and expert opinion from Novartis, Pfizer, Janssen, and AstraZeneca. JA received fees for lecturing and expert opinion from AbbVie and Pfizer. IrM received fees for lecturing and expert opinion from Novartis, Pfizer, Bristol Myers Squibb, Fusion Pharma, Janssen, AstraZeneca, and Sanofi. YV received fees for lecturing and expert opinion from Novartis and Pfizer. NM received fees for lecturing and expert opinion from Janssen, Sanofi, and Sotex. ElM received fees for lecturing and expert opinion from Novartis, Pfizer, and Amgen.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

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Case report: Long-term voluntary Tyrosine Kinase Inhibitor (TKI) discontinuation in chronic myeloid leukemia (CML): Molecular evidence of an immune surveillance

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The classical natural history of chronic myeloid leukemia (CML) has been drastically modified by the introduction of tyrosine kinase inhibitor (TKI) therapies. TKI discontinuation is currently possible in patients in deep molecular responses, using strict recommendations of molecular follow-up due to risk of molecular relapse, especially during the first 6 months. We report here the case of a patient who voluntarily interrupted her TKI therapy. She remained in deep molecular remission (MR4) for 18 months followed by detection of a molecular relapse at +20 months. Despite this relapse, she declined therapy until the occurrence of the hematological relapse (+ 4 years and 10 months). Retrospective sequential transcriptome experiments and a single-cell transcriptome RNA-seq analysis were performed. They revealed a molecular network focusing on several genes involved in both activation and inhibition of NK-T cell activity. Interestingly, the single-cell transcriptome analysis showed the presence of cells expressing NKG7, a gene involved in granule exocytosis and highly involved in anti-tumor immunity. Single cells expressing as granzyme H, cathepsin-W, and granulysin were also identified. The study of this case suggests that CML was controlled for a long period of time, potentially *via* an immune surveillance phenomenon. The role of NKG7 expression in the occurrence of treatment-free remissions (TFR) should be evaluated in future studies.

KEYWORDS

chronic myelogenous leukemia, natural killer cells, NKG7, TFR, CML

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Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm initiated by the occurrence of the Philadelphia chromosome (Ph1) in a primitive hematopoietic stem cell. The natural history of disease from a chronic phase towards accelerated and blast phases has now been drastically modified by the introduction of the targeted therapies. BCR::ABL1 tyrosine kinase inhibitors (TKIs) improved overall survival in patients with chronic phase CML (1), and recent data suggest that the life expectancy of CML patients responding to TKI could currently be similar to that of the general population of the same age and sex (2).

Up to 80% of newly diagnosed CML patients in chronic phase (CP) achieve a molecular response under TKI therapies especially by the use of second-generation TKI such as Nilotinib (3). A major issue remaining today in the CML field is the persistence of stem cells in deep molecular response due to the resistance of leukemic stem cells (LSC) to TKI (4–6). Indeed, TKI treatment is unable to eliminate quiescent stem cell fraction (7), and the survival of LSC is independent on BCR::ABL TK activity (8). In half of the CML patients in deep molecular responses, TKI discontinuation can allow a treatment-free remission (TFR) (9, 10). In several TKI-discontinuation trials, it has been shown that an immunological mechanism contributes to the absence of relapse, in particular with increased levels of NK cells (11).

We report here the case of a patient who voluntarily stopped her TKI therapy and remained in deep molecular response without therapy for 18 months. At the end of this period of 18 months, which was operationally a TFR, a molecular relapse was diagnosed, but the patient declined therapy for an additional 3 years until hematological relapse. A transcriptome analysis was performed at several time points of this TKI-discontinuation condition with low or high BCR::ABL^{IS} levels. In addition, a single-cell transcriptome analysis of the circulating mononuclear cells was performed.

Case description

A CML was diagnosed in 2014 in a 30-year-old female patient with low Sokal score. She was initially treated with Imatinib, but this drug was rapidly stopped because of intolerance, and she was switched to Dasatinib (Figure 1). A deep molecular response (RM4) was rapidly obtained with a clinical and molecular follow-up every 3 months. At January 2016, she stopped her therapy voluntarily. In August 2016, molecular analysis identified a relapse with BCR::ABL/ABL^{IS} levels, which rose to 3%. She disclosed at that time that she was not taking Dasatinib. Despite this molecular recurrence, she declined therapy while accepting regular hematological and molecular analyses. BCR::ABL^{IS} levels rose to 25% in October 2017 and to 38% in September 2019 (Figure 1). During this 3-year period of molecular progression, blood counts and clinical examinations remained normal. In October 2019, BCR::ABL^{IS} was 51%, and she was in hematological relapse. She refused bone marrow aspirate but accepted to start TKI therapy by Dasatinib 100 mg/day. After an initial response, she reduced the dose of Dasatinib to 50 mg/day because of GI intolerance and stopped it again in September 2020. In March 2021, she was again in hematological relapse with BCR::ABL^{IS} level at 59%. She accepted Dasatinib at a very low dose (20 mg/day), which induced a major molecular response (MMR) that is currently ongoing.

To gain insights into the molecular events occurring during this "self-induced" drug interruption, we have performed with the informed consent of the patient, serial transcriptome analyses in frozen peripheral blood cells (PBMCs). The first three were performed during periods of low BCR::ABL expression, whereas the last four were performed in samples with high BCR::ABL expression (Figure 1). All these analyses were performed without TKI administration. In one sample, a single-cell RNA-seq analysis was performed. For all retrospective samples for which BCR::ABL^{IS} quantification was performed, peripheral blood mononuclear cells have been processed to perform total RNA extraction and whole transcriptome experiment on microarray chip ClariomS human (Thermo Fisher Scientific).

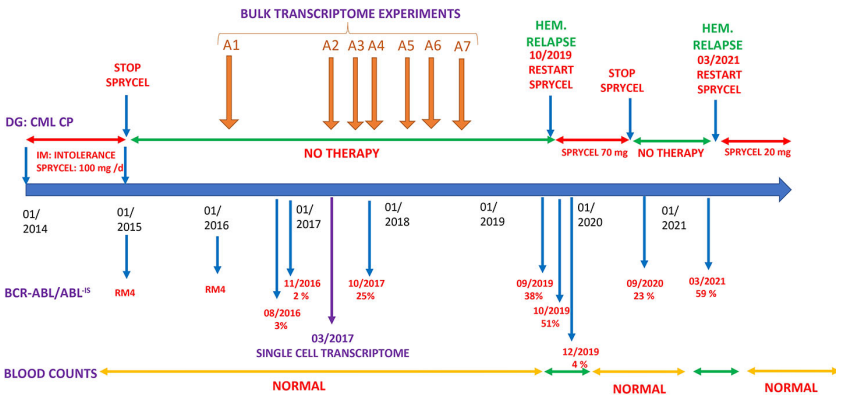

FIGURE 1
Clinical history. DG, diagnosis; IM, imatinib; Hem. relapse, hematological relapse (see text).

Discussion

Although this patient declined therapy for a long period of time (5 years), the progression of the disease was very slow. This prompted us to perform bulk transcriptome analyses from serially collected blood samples to study the expression of the genes involved during this natural history (Figure 1). For each timepoint of these samples, the results of BCR::ABL^{IS} quantifications were available. BCR::ABL-IS quantification was taken as predictor for Pavlidis Template Matching algorithm (12) to elucidate gene expression profile, which followed the amount of BCR::ABL transcript during the time course of the disease. After false discovery rate adjustment of the gene signature, 188 genes were found to be connected to BCR::ABL^{-IS} (Supplemental Table S1). One of the top associated molecule was the BCR transcript (rank = 30), suggesting a good correlation between BCR::ABL^{-IS} quantification and transcriptome time-course experiments.

Evidence of the enrichment of an immunological signature during the time out of therapy

Functional enrichment performed with time course transcriptome expression profile (Supplemental Table S1) on ToppCell Atlas database revealed that this signature mainly characterized natural killer-T (NK-T) lymphocyte cell type (Figure 2A), with specific upregulation of IL21R, ADGR1, S1PR5, AUTS2, BCR, BPGM, ANKRD16, EPHA4, OCLN, CHIC1, and CEP41 (Figure 2B). These results suggested that during the natural evolution of the disease, the molecular signature of an NK/T lymphocyte cell population could be detected in the PBMC.

A single-cell RNA-seq analysis was performed on the PBMC sample collected on March 2017 where the patient had evident molecular progression with a blood BCR::ABL^{-IS} level at 2%. She was out of therapy at that time (Figure 1), and her blood counts were normal.

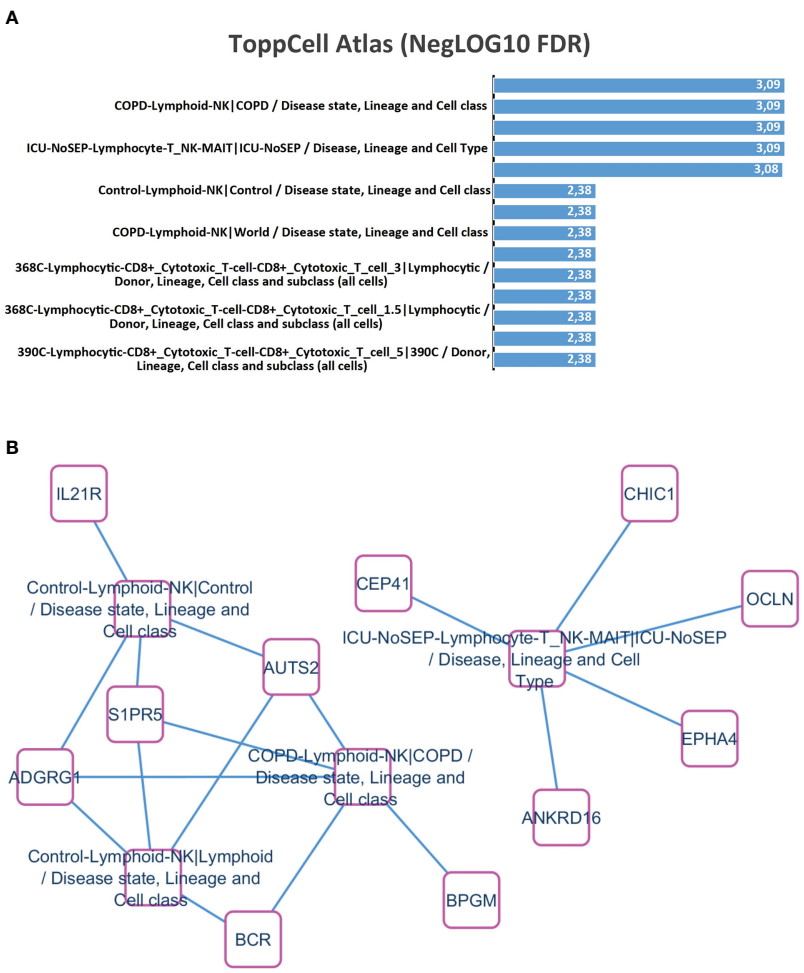

FIGURE 2
NK/T lymphocyte cell-type enrichment during time course transcriptome experiments correlated with BCR::ABL^{-IS} quantification. **(A)** Barplot of functional enrichment performed on ToppCell Atlas database (negative log10 of false discovery rate p-values); **(B)** NK-T lymphocyte cell enrichment network.

Single-cell transcriptome of 9,036 PBMC could be demultiplexed after Cell Ranger pipeline. A mean number of 9,054 transcripts has been found to be expressed by cell. After preprocessing of single-cell transcriptome of PBMCs, dimension reduction by PCA (Figure 3A) and UMAP identified three main topological cell clusters including T-lymphocytes (expressing CD3E), B-lymphocytes (expressing MS4A1), and monocytes expressing CD68 (Figure 3B). UMAP dimension reduction conjugated to unsupervised clustering identified 11 distinct cell communities (Figure 3C), which expressed distinct patterns of markers (Figure 3D). Clusters 7–10 were found to be less represented quantitatively in the blood of the patient (Figure 3D).

Clusters 0 and 1, which were the largest ones, seemed to share expression of markers (Figure 3D) corresponding to T lymphocytes (Figures 3B, C). In terms of expression specificity, cluster 5 shared some markers with clusters 0 and 1(Figure 3D). Interestingly, among the cluster of T-lymphocytes (Figures 3B, C), we could observe another cluster (cluster C3) that expressed a distinct signature from the three other T-lymphocyte clusters (0, 1, and 5, Figure 3D). Among the best markers characterized during single-cell transcriptome analysis, a high expression of NKG7 was revealed in individual cluster C3 (Figure 4A), which correspond to a subpopulation of CD3E+ T lymphocytes (Figure 4B). Differential expression analysis between the distinct cell clusters showed that a high expression of natural killer effective markers

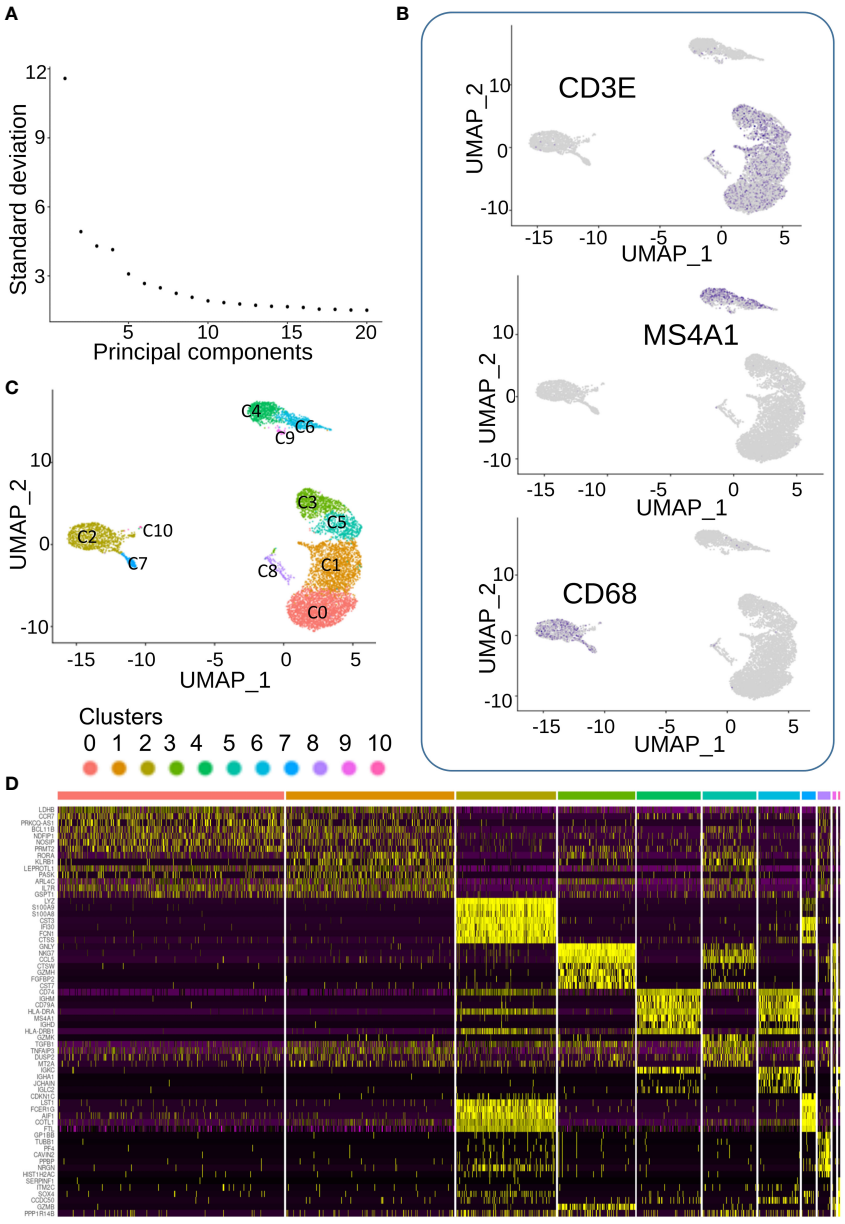

FIGURE 3
Single cell transcriptome analysis of CML blood cells. **(A)** Elbow plot of dimension reduction. **(B)** Feature plot of markers characterizing three main cell populations: CD3E for T lymphocyte, CD68 for monocytes, and MS4A1 for B lymphocytes. **(C)** Unsupervised clustering on UMAP dimension reduction. **(D)** Expression heatmap of best molecular markers of the eleven cell communities.

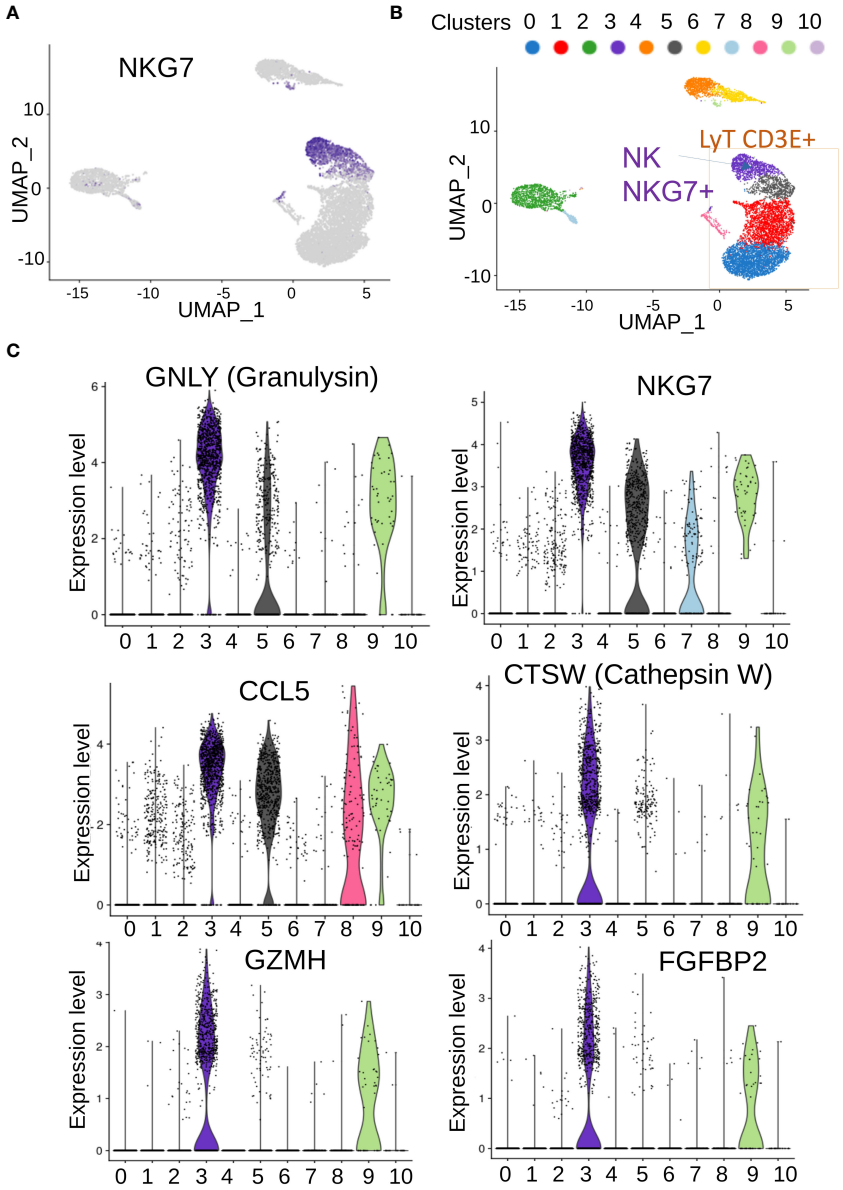

FIGURE 4
Characterization of genes associated with NK/T cells in PBMC. **(A)** UMAP of NKG7-positive cells in single-cell transcriptome; **(B)** inference of NKG7-positive cell on cells communities identified; **(C)** best over expressed markers identified in cluster 3 of NKG7-positive cells.

characterized cluster C3 (Figure 4C), such as GNLY (granulysin), GZMH (granzyme H), and CTSW (cathepsin W). In addition, a high expression of CCL5 chemokine and FGFBP2 secreted protein (cytotoxic marker) was found in cluster C3 of natural killer cells from this patient (Figure 4C). These molecular results on single-cell transcriptome performed on PBMC suggested therefore the presence of an immunologically competent cells in the blood sample analyzed.

Interestingly, functional enrichment of the gene signature on ToppCell atlas signature highlighted that activation of genes involved in natural killer/T lymphocytes following BCR::ABL quantification. NK cells play important roles in innate immunity against virus or tumors by secreting cytotoxic granules (13). In the case reported here, the transcriptomic profile was compatible with both NK and a NK/T-cell population, the latter known to exhibit anti-leukemia activity (14, 15). The deficient function of the invariant NK/T cells in CML and their restoration on TKI therapy has previously been reported (16, 17). The role of NK cells in CML immune surveillance has also been suggested in patients remaining in deep molecular response after TKI discontinuation (11).

We then analyzed genes expressed in NK cell clusters among NK-T lymphocyte population in single-cell transcriptome, while the patient was in molecular recurrence and without TKI therapy but without hematological relapse. This NK cell cluster was positive for NKG7 expression and cells highly expressed effectors of cytotoxicity genes. Granulysin is an antimicrobial peptide (AMP) highly active against cancer cells such as melanoma (18). Cathepsin W (lymphopain) is a papain-like cysteine protease of unknown function that is specifically expressed in NK cells and to a lesser extent in cytotoxic T cells (CTL), and its expression in NK-92 (NK cell model) is linked to the cytotoxicity observed against K562 (19). Granzyme H is well-known cytotoxic effector of NK cells (20). NKG7+ positive NK cell cluster also

highly expressed FGFBP2-secreted protein that is known to be a marker of the cytotoxicity of lymphocytes (21).

These molecular findings could also be linked to the immuno-molecular data obtained from the whole transcriptome analyses. Indeed, transcriptome experiments showed that the IL21R expression (receptor to interleukin 21) followed the BCR::ABL^{IS} quantification analyses during TKI interruption period. IL-21 has been shown to participate to the NK cell expansion of K562 cell line engineered to express membrane-bound IL-21 feeder in combination with IL-2 (22, 23).

S1P (5) receptor found in NK cell network is also known to be essential for human NK cell trafficking (24, 25). Another interesting receptor expression was that of GPR56, which is an inhibitory receptor of NK cells, negatively regulating effector functions such as production of inflammatory cytokines and cytolytic proteins, degranulation, and target cell killing through association with the tetraspanin CD81 (26).

Overall, the molecular analyses reported during the long-term TKI discontinuation in this patient allowed to detect major modulation of genes involved with NK cell activity. Interestingly, several genes that we identified to be part of the immunological pathway seemed to be activated with a successful TFR after TKI discontinuation (10, 27–29). Importantly we have identified for the first time NKG7 expression in single cell transcriptome from CML PBMC, this gene being a major regulator of granule exocytosis in cancer (30). Its expression deserves further evaluation in patients with CML.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by INSERM ethical committee. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the patients/participants for the publication of this case report.

Author contributions

JI, PM, NO and DC: Performed molecular Single cell experiments; CD: Bioinformatics analyses; XF performed BCR-ABL quantification experiments, JF, AB-G: validated BCR-ABL data, AT, AB-G: Clinical follow-up, plan experiments, JI, PM, CD, AB-G, AT: wrote the paper. All authors contributed to the article and approved the submitted version

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1117781/full#supplementary-material

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Treatment discontinuation following low-dose TKIs in 248 chronic myeloid leukemia patients: Updated results from a campus CML real-life study

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TKIs long-term treatment in CML may lead to persistent adverse events (AEs) that can promote relevant morbidity and mortality. Consequently, TKIs dose reduction is often used to prevent AEs. However, data on its impact on successful treatment-free remission (TFR) are quite scarce. We conducted a retrospective study on the outcome of CML subjects who discontinued low-dose TKIs from 54 Italian hematology centers participating in the Campus CML network. Overall, 1.785 of 5.108 (35.0%) regularly followed CML patients were treated with low-dose TKIs, more frequently due to relevant comorbidities or AEs (1.288, 72.2%). TFR was attempted in 248 (13.9%) subjects, all but three while in deep molecular

response (DMR). After a median follow-up of 24.9 months, 172 (69.4%) patients were still in TFR. TFR outcome was not influenced by gender, Sokal/ELTS risk scores, prior interferon, number and last type of TKI used prior to treatment cessation, DMR degree, reason for dose reduction or median TKIs duration. Conversely, TFR probability was significantly better in the absence of resistance to any prior TKI. In addition, patients with a longer DMR duration before TKI discontinuation (i.e., >6.8 years) and those with an e14a2 *BCR::ABL1* transcript type showed a trend towards prolonged TFR. It should also be emphasized that only 30.6% of our cases suffered from molecular relapse, less than reported during full-dose TKI treatment. The use of low-dose TKIs does not appear to affect the likelihood of achieving a DMR and thus trying a treatment withdrawal, but might even promote the TFR rate.

KEYWORDS

chronic myeloid leukemia, tyrosine kinase inhibitors, treatment-free remission, low dose, resistance, outcome

1 Introduction

The therapeutic armamentarium of chronic myeloid leukemia (CML) has greatly improved after small molecule tyrosine kinase inhibitors (TKIs) targeting *BCR::ABL1* became available. The 10-year survival rate in CML-chronic phase increased from about 20% to 80%–90%, allowing for near-normal life expectancy (Bower et al., 2016).

While highly effective, these drugs also have an often mild to moderate, but sometimes severe, toxicity profile. Indeed, long-term treatment with TKIs may lead to chronic adverse events (AEs) that can negatively affect patients' quality of life (QoL) and can promote relevant morbidity and mortality. In particular, there has been increasing evidence of more serious AEs with second- or third-generation TKIs, such as pleural effusion and pulmonary arterial hypertension with dasatinib (Campbell and Copland, 2013), hyperglycemia, dyslipidemia and arterial occlusive events (AOEs) with nilotinib (Emir et al., 2013; Hiwase et al., 2013), gastrointestinal toxicities with bosutinib (Cortes et al., 2012), and hypertension, AOEs and pancreatic dysfunction with ponatinib (Cortes et al., 2018).

Dose reductions due to AEs are part of the daily management of TKIs (Copland, 2019): often these dose reductions are kept stable over the long term, particularly in patients who have already achieved a durable response. This optimization of the TKI dose is related to better adherence, improved QoL and in most cases do not jeopardize a stable response once achieved, thus confirming, as studies on intermittent TKI treatment have already shown (Russo et al., 2013; Malagola et al., 2021), that responding patients are often overtreated. Furthermore, as also demonstrated by the UK DESTINY study (Clark et al., 2017; Clark et al., 2019) and real-world data from Hammersmith Hospital (Claudiani et al., 2021), a TKI de-escalation strategy, in addition to not compromising the possibility of treatment discontinuation, could also improve the success of treatment-free remission (TFR) protocols.

However, while discontinuing therapy appears as a safe option for approximately half of the patients obtaining an optimal response (Ross and Hughes, 2020), so far, data on the impact of long-term TKI dose de-escalation on successful TFR are rather scarce.

The aim of our study was therefore to evaluate the propensity of Italian hematologists to attempt TFR in CML patients treated with low-dose TKIs, both at diagnosis and during follow-up.

2 Methods

We conducted a retrospective analysis on the outcome of CML patients who discontinued low-dose TKIs between May 2010 and September 2022 from 54 Italian hematology centers participating to the "Campus CML", an active research network of physicians involved in CML management throughout Italy, with the aim of investigating different aspects of the disease (Iurlo et al., 2022).

Each center was asked to complete an online survey which included questions regarding the use of low-dose TKIs in real-life clinical practice and the willingness of physicians to offer TFR even to subjects treated with reduced doses of TKI for AEs and/or relevant comorbidities or to patients who have already achieved an optimal molecular response.

The following key disease characteristics were then required for each patient attempting TFR after low-dose TKI treatment: socio-demographic variables, risk scores (i.e., Sokal and ELTS), all treatments (including interferon) before and after discontinuation, duration of each treatment, reasons for dose reduction, and best response to each treatment.

Molecular monitoring and classification of responses were defined according to current ELN recommendations (Hochhaus et al., 2020): in particular, major molecular response (MMR) as a *BCR::ABL/ABL* ratio ≤ 0.1%, and deep molecular response (DMR) as MR4 (*BCR::ABL1/ABL* ratio ≤ 0.01%), MR4.5 (*BCR::ABL1/ABL* ratio ≤ 0.0032%), or MR5 (*BCR::ABL1/ABL* ratio ≤ 0.001%). Molecular relapse was defined as loss of MMR. Consequently, TFR was calculated from TKI discontinuation to loss of MMR.

Follow-up began on the day of TKI discontinuation and ended at the earliest of molecular relapse or 31 December 2022. As only 65 (26.2%) patients were followed up for more than 4 years and no molecular recurrence was observed among them, we truncated follow-up time at 4 years.

We analyzed time since to molecular relapse based on selected variables using the Kaplan-Meier survivor function

TABLE 1 Baseline demographics and clinical characteristics of 248 CML patients.

Characteristics	Patients (N = 248)
M/F	110/138
Age at CML diagnosis (years), median (range)	49.3 (19.1–81.4)
Sokal risk, n (%)	
Low	122 (49.2)
Intermediate	78 (31.5)
High	33 (13.3)
NA	15 (6.0)
ELTS risk, n (%)	
Low	181 (73.0)
Intermediate	37 (14.9)
High	13 (5.2)
NA	17 (6.9)
BCR::ABL1 p210 transcript type, n (%)	
e14a2	155 (62.5)
e13a2	56 (22.6)
e14a2/e13a2	19 (7.7)
Other types	8 (3.2)
NA	10 (4.0)
Previous IFN, n (%)	39 (15.7)
Time from diagnosis to TKI discontinuation (months), median (range)	115.2 (16.0–355.9)
Duration of TKI therapy (months), median (range)	111.2 (16.0–252.1)
Duration of low-dose TKI (months), median (range)	37.4 (0.6–197.9)
Age at TKI discontinuation (years), median (range)	61.9 (29.7–89.2)
MR at TKI discontinuation, n (%)	
MMR	3 (1.2)
MR4.0	55 (22.2)
MR4.5	96 (38.7)
MR5.0	94 (37.9)
Duration of DMR before TKI discontinuation (months), median (range)	65.0 (3.0–186.0)

Abbreviations: CML, chronic myeloid leukemia; NA, not available; MR, molecular response; MMR, major molecular response; DMR, deep molecular response.

and the log-rank-test. Statistical analyses were performed with Stata 17 (StataCorp. 2021).

3 Results

Overall, 1.785 of 5.108 (35.0%) regularly followed CML-chronic phase patients were treated with low-dose TKIs. More specifically, a TKI dose reduction was reported in 727 (27.5%) out of 2.648 patients treated with imatinib, 362/1.133 (32.0%) with nilotinib, 304/813 (37.4%) with dasatinib, 213/294 (72.5%) with bosutinib and 179/220 (81.4%) with ponatinib. The TKI dose was reduced in the majority of patients (1.288, 72.2%) due to AEs (916, 51.3%) or relevant comorbidities (372, 20.8%), while in the remaining subjects (497, 27.8%) TKIs were reduced for physicians' decision, after obtaining a stable molecular response (MMR/DMR), with the aim of preventing the development of AEs, thereby improving TKIs tolerability. No progression toward advanced-phase disease was observed during treatment with low-dose TKIs.

TFR was attempted in 248 (13.9%) out of 1.785 patients, 138 of whom were female and 110 were male, with a median age at CML diagnosis of 49.3 years (Table 1). As expected, dose reduction was due to AEs in more than half of the cases (149 patients, 60.1%), while in the remaining subjects TKIs were reduced after reaching an optimal molecular response

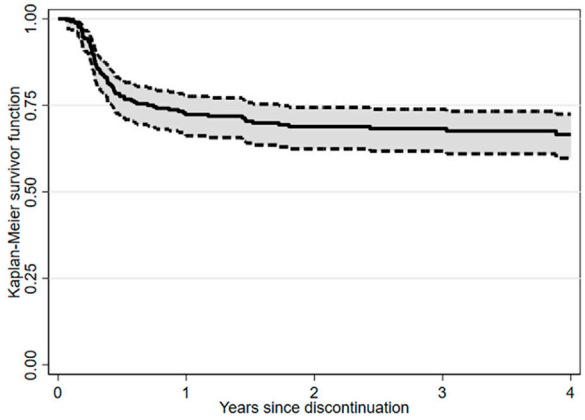

FIGURE 1
Treatment-free remission (TFR) after tyrosine kinase inhibitor (TKI) therapy (Kaplan-Meier survivor function).

(Supplementary Table S1). Interestingly, 47 (18.9%) patients experienced TKI resistance before treatment discontinuation, in the absence of any *BCR::ABL1* kinase domain mutation.

The most widely used low-dose TKI before attempting TFR was imatinib (99 patients out of 727–13.6%), followed by nilotinib (90 patients out of 362–24.9%), dasatinib (51 patients out of 304–16.8%) and, to a lesser extent, ponatinib (five patients out 179–2.8%) and bosutinib (three patients out of 213–1.4%). Overall, 117 (47.2%) patients experienced a ≥ 50% reduction in TKI dose from baseline (Supplementary Table S2).

Before attempting TFR, 152 (61.3%) patients were treated with a single TKI, while 80 and 16 patients had received second or later-line therapy, respectively, with a median TKI treatment duration of 111.2 months (range, 16.0–252.1). At the time of TFR, 245 (98.8%) patients had already achieved a DMR, with a median duration of 65.0 months (range, 3.0–186.0).

After a median follow-up from TKIs discontinuation of 24.9 months (range, 1.1–149.2), 172 (69.4%) patients were still in TFR (Figure 1). Interestingly, TFR outcome was not affected by any of the following parameters (Supplementary Table S3): gender, Sokal or ELTS risk scores, prior interferon treatment, number and last type of TKIs used before discontinuation of therapy, degree of DMR (i.e., MR4 vs. MR4.5 or better), reason for dose reduction (i.e., AEs vs. DMR achievement) or median duration of therapy with TKIs (Supplementary Figures S1, S2). In contrast, TFR was markedly better in the absence of a history of resistance to any previous TKI (Table 2; Figure 2); furthermore, as expected, patients with a longer DMR duration (i.e., >6.8 years) (Supplementary Figure S2) and those with an e14a2 *BCR::ABL1* transcript type showed a trend toward prolonged TFR (Table 2).

Seventy-six (30.6%) patients experienced molecular recurrence (≤MMR) after a median time from treatment interruption of 4.1 months (range, 0.8–47.3), representing an early relapse (within 6 months) in 54/76 patients (71.1%). All of these cases who had a molecular relapse restarted the same dose of TKI treatments they had before the TFR attempt and regained at least an MMR within 8 months of TKI resumption.

4 Discussion

In this large, real-life series of CML patients regularly followed in Italy, TKIs treatment at a reduced dose represents an important reality, being bosutinib and ponatinib the drugs that most frequently required a dose reduction. More notably, TKI treatment has usually been initiated at full dose (excluding the elderly or patients with relevant comorbidities), with nearly 30% of TKI dose reductions performed in clinical practice with the goal of long-term treatment improvement once an optimal molecular response has been obtained, while at the same time preventing the development of AEs, thus improving TKIs tolerability.

Indeed, it should be emphasized that TKI dose optimization should be taken into account from the outset as, after the development of chronic toxicities, the true possibility of complete resolution of AEs is still a matter of debate, especially in some specific contexts (Okamoto et al., 2020).

In fact, while all the information on the prescription of TKIs used in CML treatment indicate a starting dose and rules and suggestions for transiently suspending or reducing the dose in case of clinical and biochemical AEs, suggesting a return to the ideal dose once the AEs disappear or alleviate, in daily clinical practice many physicians prescribe TKI starting dose lower than those foreseen in the product information in case of patients of advanced age or with relevant comorbidities. Furthermore, when an AEs improves or disappears, the TKI is maintained at a reduced dose over the long term, essentially to avoid recurrence of the same AE or, generically, to promote a better QoL and to ensure or reassure a good adherence, thus allowing a broader use of even those second- or third-generation TKIs not adequately indicated in the presence of specific comorbidities (Iurlo et al., 2021).

Furthermore, while most studies of TFR in CML subjects abruptly discontinued TKIs, more recently some authors have evaluated a new treatment strategy based on gradual therapy withdrawal before TKI interruption in the context of clinical trials or real-life experiences (Clark et al., 2017; Clark et al., 2019; Cayssials et al., 2020; Claudiani et al., 2021): among others, the DESTINY study examined TKI de-escalation treatment in 174 patients with stable MMR or MR4, after at least 3 years of treatment with full-dose imatinib, nilotinib, or dasatinib (Clark et al., 2017). There was no progression or cytogenetic recurrence, with monthly monitoring allowing rapid identification of all cases of molecular relapse. Furthermore, chronic AEs improved in most cases (Clark et al., 2019).

More recently, the prospective, multicenter phase II DANTE study (NCT03874858) aimed to evaluate the safety of first-line nilotinib de-escalation and its impact on TFR success in Italian CML-chronic phase subjects. While in a previously interim analysis 1 year of nilotinib de-escalation prior to TFR in patients with stable DMR was shown to be safe and effective (Breccia et al., 2021), a molecular recurrence rate of approximately 32% 1 year after stopping nilotinib was reported, thus demonstrating that de-escalation of this drug before attempting TFR may be a successful dose optimization strategy (Stagno et al., 2022).

Accordingly, we then evaluated the potential effect of low-dose TKIs on TFR outcome in a large real-life series of 248 CML-chronic phase patients managed in Italy.

TABLE 2 Molecular recurrence risk and hazard ratios from univariate Cox models according to selected variables.

	N. patients	N. molecular recurrence (%)	HRs	95% CI	*p*
Gender					
M	110	30 (27.3)	1.00	Reference	
F	138	46 (33.3)	1.28	0.81–2.02	0.30
Sokal risk					
Low	122	39 (32.0)	1.00	Reference	
Intermediate	78	20 (25.6)	0.81	0.47–1.38	0.43
High	33	15 (45.4)	1.55	0.86–2.82	0.15
ELTS risk					
Low	181	58 (32.0)	1.00	Reference	
Intermediate	37	13 (35.1)	1.11	0.61–2.03	0.73
High	13	3 (23.1)	0.73	0.23–2.34	0.60
Transcript type					
e14a2	155	44 (28.4)	1.00	Reference	
e13a2	56	22 (39.3)	1.65	0.99–2.75	**0.06**
e14a2/e13a2	19	7 (36.8)	1.36	0.61–3.03	0.44
Previous IFN					
NO	209	67 (32.1)	1.00	Reference	
YES	39	9 (23.1)	0.62	0.31–1.24	0.18
First-line TKI					
Imatinib	177	54 (30.5)	1.00	Reference	
Dasatinib	20	10 (50.0)	1.73	0.88–3.41	0.11
Nilotinib	51	12 (23.5)	0.81	0.43–1.51	0.50
Reason for dose reduction					
MR achievement	100	32 (32.0)	1.00	Reference	
AEs	148	44 (29.7)	0.87	0.55–1.37	0.55
Duration of TKIs (years)					
<10	142	45 (31.7)	1.00	Reference	
≥10	106	31 (29.2)	0.89	0.56–1.40	0.60
Last TKI at discontinuation					
Imatinib	99	27 (27.3)	0.75	0.47–1.21	0.24
2/3G-TKIs	149	49 (32.9)	1.00	Reference	
Line of therapy at discontinuation					
First-line	152	45 (29.6)	0.82	0.52–1.30	0.41
Second- or later lines	96	31 (32.3)	1.00	Reference	
Resistance to previous TKIs					
YES	47	24 (51.1)	1.00	Reference	
NO	201	52 (25.9)	2.45	1.51–3.98	**0.000**
DMR degree at TFR					
MR4.0	55	18 (32.7)	1.00	Reference	
≥MR4.5	190	56 (29.5)	0.86	0.50–1.46	0.57
Duration of DMR (years)					
<4.2	84	32 (38.1)	1.00	Reference	
4.2–6.7	81	24 (29.6)	0.71	0.42–1.21	0.21
≥6.8	83	20 (24.1)	0.57	0.33–0.99	**0.05**

Abbreviations: M, male; F, female; MR, molecular response; AEs, adverse events; DMR, deep molecular response; HR, hazard ratio; CI, confidence interval. Bold values are statistically significant.

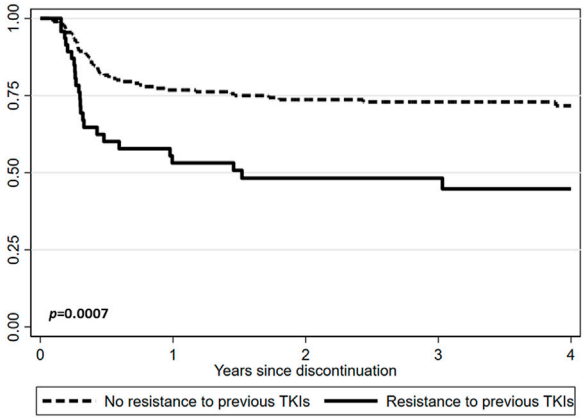

FIGURE 2
Treatment-free remission (TFR) after tyrosine kinase inhibitor (TKI) therapy (Kaplan-Meier survivor function) according to resistance to previous TKIs.

In this context, as already reported in a previous paper (Iurlo et al., 2022), we confirm in a larger patients' population that the use of low-dose TKIs does not seem to affect the possibility of achieving a DMR and therefore attempting a suspension of therapy.

While being aware of the limitations of this study, mainly represented by its retrospective nature, it should be emphasized that only 30.6% of our cases suffered from molecular relapse, less than reported in patients previously treated with full-dose TKIs (Hernández-Boluda et al., 2018; Fava et al., 2019).

In addition, we have now demonstrated that the only clinical variable associated with molecular recurrence was resistance to prior TKIs (Figure 2), thus confirming observations from previous studies, for example, the DADI trial in which patients who switched to dasatinib because of resistance to imatinib had worse outcomes than those who switched for other reasons (Imagawa et al., 2015). Similarly, in the more recent STOP 2G-TKI French study a history of suboptimal response or resistance to imatinib prior to dasatinib or nilotinib was the only baseline factor associated with significantly worse TFR (Rea et al., 2017). Consequently, it must be admitted that in this context only a select subset of patients has the possibility of achieving a successful TFR, probably for a less aggressive disease.

In fact, approximately 14.0% of our patients treated with TKIs at a reduced dose were considered eligible to discontinue therapy, thus demonstrating that TFR was not compromised using low-dose TKIs. Furthermore, treatment dose adjustments have important prognostic implications even in those patients who do not wish to attempt TFR, with the clinical potential to reduce both therapy-related AEs and overall treatment costs without jeopardizing the possibility of maintaining a good response.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

AI conceptualized and designed the study. AI, DCa, FC, MM, GB, MBn, GR-C, MT, FL, AG, PP, EA, IC, CB, MP, SA, MI, ES, GL, AM, SR, CE, AS, AT, RL, GC, MBc, SG, LL, CF, RF, GS, GR, and MBr. collected and assembled the data. DCo performed the statistical analysis. AI and DCa wrote the manuscript. RF, GS, GR, and MBr. critically reviewed the manuscript. AI, DCa, DCo, FC, MM, GB, MBn, GR-C, MT, FL, AG, PP, EA, IC, CB, MP, SA, MI, ES, GL, AM, SR, CE, AS, AT, RL, GC, MBc, SG, LL, CF, RF, GS, GR, and MBr approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1154377/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Treatment-free remission (TFR) after tyrosine kinase inhibitor (TKI) therapy (Kaplan-Meier survivor function). **(A)** TFR according to gender. **(B)** TFR according to pretreatment with interferon. **(C)** TFR according to Sokal risk score. **(D)** TFR according to TKIs used before therapy cessation.

SUPPLEMENTARY FIGURE S2
Treatment-free remission (TFR) after tyrosine kinase inhibitor (TKI) therapy (Kaplan-Meier survivor function). **(A)** TFR according to *BCR::ABL1* transcript type. **(B)** TFR according to lines of therapy. **(C)** TFR according to median duration of TKIs therapy. **(D)** TFR according to median duration of DMR. **(E)** TFR according to the degree of DMR. **(F)** TFR according to the main reason for TKIs dose reduction.

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Prospective monitoring of chronic myeloid leukemia patients from the time of TKI discontinuation: the fate of peripheral blood CD26$^+$ leukemia stem cells

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Introduction: In chronic myeloid leukemia (CML), about half of the patients achieving a deep and stable molecular response with tyrosine kinase inhibitors (TKIs) may discontinue TKI treatment without disease recurrence. As such, treatment-free remission (TFR) has become an ambitious goal of treatment. Given the evidence that deepness and duration of molecular response are necessary but not sufficient requisites for a successful TFR, additional biological criteria are needed to identify CML patients suitable for efficacious discontinuation. Leukemia stem cells (LSCs) are supposed to be the reservoir of the disease. Previously, we demonstrated that residual circulating CD34+/CD38-/CD26+ LSCs were still detectable in a consistent number of CML patients during TFR.

Methods: CML LSCs could be easily identified by flow-cytometry as they express the CD34+/CD38-/CD26+ phenotype. In this study, we explored the role of these cells and their correlation with molecular response in a cohort of 109 consecutive chronic phase CML patients prospectively monitored from the time of TKI discontinuation.

Results: After a median observation time of 33 months from TKI discontinuation, 38/109 (35%) patients failed TFR after a median time of 4 months, while 71/109 (65%) patients are still in TFR. At TKI discontinuation, peripheral blood CD26+LSCs were undetectable in 48/109 (44%) patients and detectable in 61/109 (56%). No statistically significant correlation between detectable/undetectable CD26+LSCs and the rate of TFR loss was found (p = 0.616). The incidence of TFR loss based on

the type of TKI treatment was statistically significant for imatinib treatment compared to that of nilotinib (p = 0.039). Exploring the behavior of CD26+LSCs during TFR, we observed fluctuating values that were very variable between patients, and they were not predictive of TFR loss.

Discussion: Up to date, our results confirm that CD26+LSCs are detectable at the time of TKI discontinuation and during TFR. Moreover, at least for the observation median time of the study, the persistence of "fluctuating" values of residual CD26+LSCs does not hamper the possibility to maintain a stable TFR. On the contrary, even patients discontinuing TKI with undetectable CD26+LSCs could undergo TFR loss. Our results suggest that factors other than residual LSCs "burden" playing an active role in controlling disease recurrence. Additional studies evaluating CD26+LSCs' ability to modulate the immune system and their interaction in CML patients with very long stable TFR are ongoing.

KEYWORDS

chronic myelogenous leukemia, treatment free remission, CD26+, leukemia stem cell, TKI, flow cytometry

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm that is still considered a model disease in cancer for its peculiar biological and clinical characteristics and for having pioneered target therapies and precision medicine approaches (Melo and Barnes, 2007; Minciacchi et al., 2021).

The development of several BCR::ABL1 tyrosine kinase inhibitor (TKI) generations, including imatinib, nilotinib, and dasatinib, currently approved as front-line therapy, revolutionized the dismal prognosis of CML patients, offering a life expectancy similar to that of the general population (Gambacorti-Passerini et al., 2011; Viganò et al., 2014; Bower et al., 2016; Hehlmann et al., 2017; Inzoli et al., 2022). However, side effects, possible correlated toxicity, and not negligible costs of lifelong TKI therapy represent still important issues regarding this disease (Efficace et al., 2011; Williams et al., 2013; Guérin et al., 2014). Today, the expectations rising for CML patients aim both to achieve long-term survival and to stop treatment (Saifullah and Lucas, 2021). It is demonstrated that a fraction of CML patients in sustained deep molecular response (DMR) may discontinue TKI treatment, and about 50% of them will maintain a treatment-free remission (TFR) without recurrence of CML (Bocchia et al., 2018). However, different aspects need to be taken into account regarding CML treatment discontinuation, as overall, just 20% of newly diagnosed CML patients will achieve a successful TFR (Atallah and Sweet, 2021; Chen et al., 2021; Pavlovsky et al., 2021). Attempting TFR is currently recommended for chronic phase (CP) CML patients on TKI treatment for at least 3 years, with a sustained DMR for at least 2 years and without evidence of any ABL kinase domain mutation (Atallah and Sweet, 2021; Pavlovsky et al., 2021). Most studies on TKI discontinuation reported that longer TKI treatment duration is associated with more chance of TFR success (Mahon et al., 2010; Saussele et al., 2018; Fujisawa et al., 2019; Molica et al., 2020; Shah et al., 2020; Richter et al., 2021), and this is strictly linked to DMR duration: CML patients with longer DMR have a lower risk of relapse (Baccarani et al., 2019; Fava et al., 2019; Castagnetti et al., 2021; Iurlo et al., 2022). The preexistence of TKI resistance must be considered, since patients who failed a treatment could also have higher chances of TFR failure, while a prior interferon-alpha (INF-α) treatment, in combination with low-risk factors and TKI therapy, may help to achieve DMR, activating quiescent cells and exposing them to the TKIs' killing effect (Chen et al., 2021; Puzzolo et al., 2022). The immune system also appears to have a predictive role in higher TFR success since it has been suggested that higher levels of NK cells may be able to eradicate Leukemia Stem Cells (LSCs) and potentiate adaptive immune responses (Hughes and Yong, 2017; Ureshino et al., 2017; Irani et al., 2020; Hsieh et al., 2021). Some studies focused attention on the type of BCR::ABL1 transcript, hypothesizing that the e13a2 transcript has higher risks of molecular relapse compared to e14a2 (Claudiani et al., 2017; Chen et al., 2022). Instead, in terms of prognostic scores, a low-risk Sokal score has better progression-free survival (PFS) and overall survival (OS), with high TFR rate, while the ELTS score could not be predictive of TFR success. Some attempts have also been made to investigate the correlation of TFR success with CML patients' age, but it is still uncertain why older patients seem to achieve higher chances of TFR compared to younger ones (Breccia et al., 2020; Inzoli et al., 2022). The hypothesis is related to the presence of LSCs, which could be less functional in older patients as for HSCs, thus reducing the risks of relapse (Saifullah and Lucas, 2021).

LSCs are considered the reservoirs of CML; their persistence is correlated to the fact that they are largely quiescent and can resist and escape TKIs' action using kinase-independent mechanisms (Chen et al., 2021).

Great efforts are ongoing to target these cells, with the aim to eradicate this silent disease fraction and to improve patients' outcomes (Cea et al., 2013; Shah and Bhatia, 2018; Baquero et al., 2019). In the previous years, several studies, including research conducted by our group, investigated CML-specific LSCs in bone marrow (BM) and peripheral blood (PB) samples (Bocchia et al., 2018). As documented, CML LSCs reside within the CD34+/CD38−/Lin− fraction and are characterized by an aberrant expression of specific surface markers, such as IL1RAP, CD25, CD93, and CD26 (DPPV) (Raspadori et al., 2019). In particular, CD26 (dipeptidylpeptidase IV) is considered a potential biomarker for the identification and quantification of CML LSCs, able to discriminate CML LSCs from normal hematopoietic stem

TABLE 1 Clinical data of the whole cohort, patients with TFR loss, and patients with TFR still sustained. WBC: white blood cells; LY: lymphocyte count; TFR: treatment-free remission; MR: molecular response; TKI: tyrosine kinase inhibitor; yrs: years; and mos: months.

Patient's characteristics	Whole cohort (*n* = 109)	TFR loss (*n* = 38)	TFR sustained (*n* = 71)
Median age at diagnosis (range)	53 years (19–76 years)	51.5 years (27–76 years)	54 years (19–76 years)
Sex			
Male	62 (57%)	20 (53%)	42 (59%)
Female	47 (43%)	18 (47%)	29 (41%)
Median WBC at discontinuation (range)	6000/mmc (3342–12680/mmc)	6000/mmc (3767–12680/mmc)	6040/mmc (3342–9,800/mmc)
Median LY (range)	1680,5/mmc (1000–3465/mmc)	1680,5/mmc (1000–3465/mmc)	1700/mmc (1000–3400/mmc)
Sokal score			
High	16 (14.5%)	4 (10.5%)	12 (17%)
Intermediate	38 (35%)	12 (31.5%)	26 (36.5%)
Low	49 (45%)	20 (53%)	29 (41%)
Unknown	6 (5.5%)	2 (5%)	4 (5.5%)
EUTOS score			
High	15 (14%)	4 (10.5%)	11 (15.5%)
Intermediate	6 (5.5%)	4 (10.5%)	2 (3%)
Low	82 (75%)	28 (74%)	54 (76%)
Unknown	6 (5.5%)	2 (5%)	4 (5.5%)
BCR::ABL1 transcript			
b2a2	27 (24.8%)	11 (28.9%)	16 (22.5%)
b3a2	54 (49.6%)	18 (47.4%)	36 (50.7%)
b2a2/b3a2	7 (6.4%)	2 (5.3%)	5 (7.1%)
b2a2/b3a3	1 (0.9%)	1 (2.6%)	-
Absent	1 (0.9%)	-	1 (1.4%)
Unknown	19 (17.4%)	6 (15.8%)	13 (18.3%)
Additional cytogenetic abnormalities	3 (2.8%)	1 (2.6%)	2 (2.8%)
Median time to complete cytogenetic response (range)	3 months (2–21 months)	3 months (2–21 months)	3 months (2–18 months)
Molecular Response after starting therapy 12 months			
MR ≤ 3	56 (51.4%)	15 (39.5%)	41 (57.7%)
MR = 4	16 (14.7%)	8 (21.1%)	8 (11.3%)
MR = 4,5	14 (12.8%)	8 (21.1%)	6 (8.5%)
MR = 5	16 (14.7%)	4 (10.5%)	12 (16.9%)
Unknown	7 (6.4%)	3 (7.8%)	4 (5.6%)
TKI therapy before discontinuation			
Imatinib	39 (35.8%)	19 (50.0%)	20 (28.2%)
Nilotinib	49 (45%)	11 (28.9%)	38 (53.5%)
Dasatinib	21 (19.2%)	8 (21.1%)	13 (18.3%)
Median TKI treatment duration (range)	7 years (3–18 years)	7 years (7–18 years)	7.5 years (3–17 years)

(Continued on following page)

TABLE 1 (*Continued*) Clinical data of the whole cohort, patients with TFR loss, and patients with TFR still sustained. WBC: white blood cells; LY: lymphocyte count; TFR: treatment-free remission; MR: molecular response; TKI: tyrosine kinase inhibitor; yrs: years; and mos: months.

Patient's characteristics	Whole cohort (*n* = 109)	TFR loss (*n* = 38)	TFR sustained (*n* = 71)
Median TKI treatment duration according to TKI			
Imatinib	8 years (4–18 years)	8 years (4–18 years)	8 years (4–16 years)
Nilotinib	7 years (3–17 years)	7 years (3–12 years)	7 years (3–17 years)
Dasatinib	8 years (3–15 years)	8 years (4–14 years)	8 years (3–15 years)
Median observation time (range)	33 months (2–63 months)	35 months (4–60 months)	32 months (2–63 months)

TABLE 2 Combination between detectable/undetectable CD26+LSC and detectable/undetectable BCR::ABL1 in relapsed/non-relapsed patients at the time of TKI discontinuation. LSCs: leukemia stem cells; TFR: treatment-free remission.

	CD26+LSCs detectable	CD26+LSCs undetectable	CD26+LSCs detectable	CD26+LSCs undetectable
	BCR::ABL1 detectable	BCR::ABL1 undetectable	BCR::ABL1 undetectable	BCR::ABL1 detectable
Patients (*n* = 109)	25/109 (22.9%)	21/109 (19.3%)	36/109 (33.0%)	27/109 (24.7%)
TFR LOSS (*n* = 38)	11/38 (29.0%)	8/38 (21.0%)	12/38 (31.6%)	7/38 (18.4%)
TFR SUSTAINED (*n* = 71)	14/71 (19.7%)	13/71 (18.3%)	24/71 (33.8%)	20/71 (28.2%)

cells from LSCs of other myeloid neoplasms (Galimberti et al., 2018; Sicuranza et al., 2022).

We earlier demonstrated the feasibility of PB CD26+LSC flow cytometry detection in CML patients and documented, in a cross-sectional study, the presence of circulating CML LSCs in a substantial number of patients in DMR both during TKI treatment and during TKI discontinuation (Bocchia et al., 2018). In order to fine-tune and further explore any active role of this staminal CML reservoir, we conducted a multicenter study with the intent to prospectively measure residual CD26+LSCs in CP-CML patients attempting TFR from the time of TKI discontinuation for a minimum of 12 months or until disease relapse, if any.

Specifically, the aims of this study were 1) to evaluate prospectively if a CML LSC "threshold" at the time of discontinuation may have an impact in predicting a successful TFR, 2) to investigate prospectively the behavior of residual LSCs along TFR, and 3) to exploit any correlation between CML LSC persistence, molecular response (MR), and TFR maintenance.

Materials and methods

Patient cohort

Consecutive CP-CML patients in sustained DMR meeting standard criteria for attempting TKI discontinuation entered this multicenter study (10 Italian hematology centers involved).

Each enrolled patient was evaluated for PB CD26+LSCs at TKI discontinuation (baseline), at +1, +2, +3, +6, and +12 months of TFR and at disease recurrence. Additionally, +18 and +24 months of TFR have been proposed as optional evaluations.

All centers provided clinical data about disease history, risk scores (EUTOS, SOKAL), cytogenetic alterations, BCR::ABL1 transcript type, line of treatment and duration of TKI therapy, duration of DMR, and MR data for each time point of the study and at the time of molecular relapse. The study was approved by each center's ethical committee, and all subjects signed an informed consent to participate in the study in accordance with the Declaration of Helsinki and each institution's policy.

Flow cytometry analysis

Six milliliters of EDTA PB samples have been collected and centrally analyzed within 24 h at the Siena Flow Cytometry lab. For all subjects, CD26 expression has been evaluated by a standardized multiparametric flow cytometry analysis on CD45+/CD34+/CD38- populations using a four-color staining protocol with the lyse stain wash procedure. Red cell lysis was performed with ammonium chloride (BD Biosciences), 1:10 diluted in deionized water, using the Lyse Wash Assistant instrument (BD Biosciences). After lysis, 2.0×10^6 leukocytes were incubated with a custom-made lyophilized pre-titrated antibody mixture 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 and a BD stain control tube lacking CD26. To reach a sensitivity of 10^{-5}, acquisition and analysis of at least 1.0×10^6 cells was performed in all samples by using the FACSCanto II and FACSLyrics flow cytometer using DIVA 8 and FACSuite software (BD, Biosciences). Additionally, to ensure reproducible results over time, we followed a standardized protocol that implied adjustments of FACS internal parameters, using the CS&T System (BD Biosciences), to keep constant the instrument performance by correcting wear of lasers and fluidic instability. The median absolute number of $CD26^+$ cells/μL has been calculated as follows: [(no. WBCs/μL) × (% of CD34+/CD38−/CD26+ on CD45+cells)] (Raspadori et al., 2019).

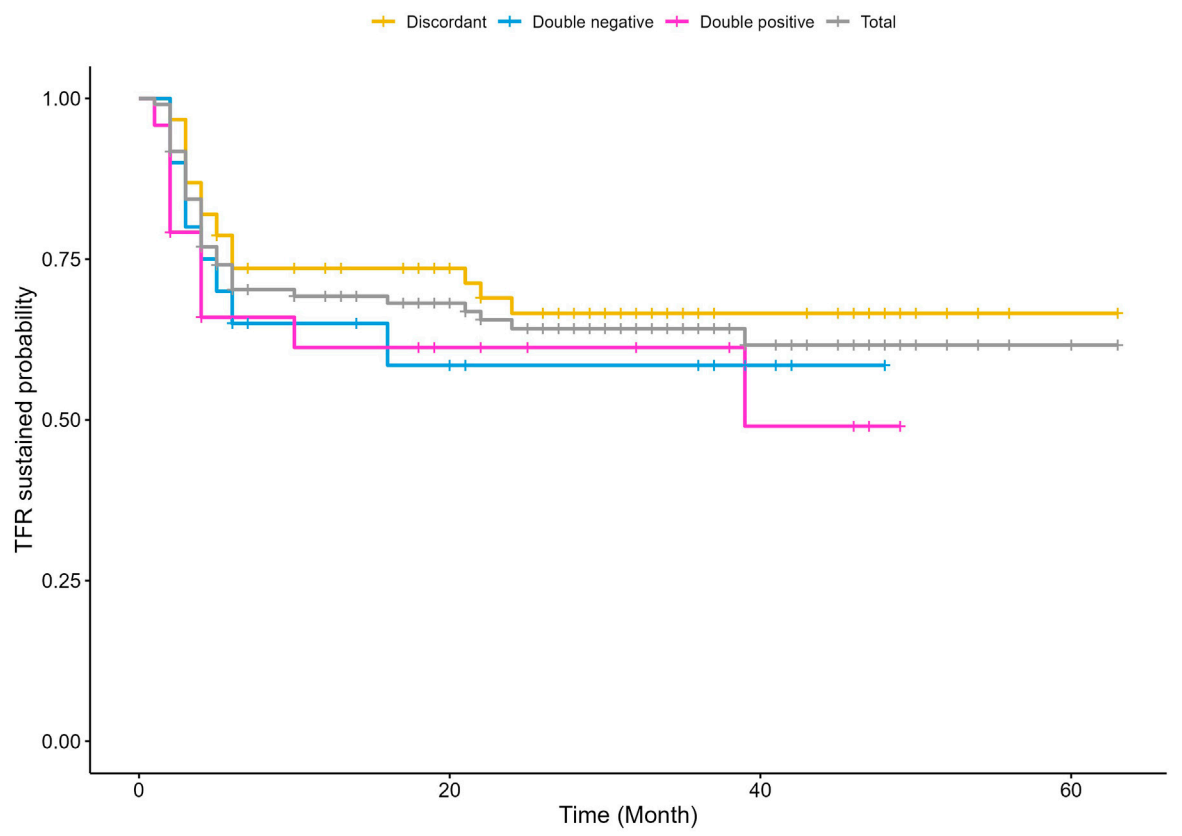

FIGURE 1
Kaplen–Meier (KM). The survival without molecular relapse is shown in CML patients' scoring, at the time of TKI discontinuation, "positive" for both CD26+LSCs and BCR::ABL1 copies (i.e., double positive), negative for both CD26+LSCs and BCR::ABL1 copies (i.e., double negative), and "discordant" with either way positive only for CD26+LSCs or BCR::ABL1 (i.e., discordant). In grey is reported the global CML patients' survival without molecular relapse.

TABLE 3 PB CD26+LSCs and BCR::ABL1 transcript at the time of TKI discontinuation, according to treatment. TFR: treatment-free remission.

Type of TKI before discontinuation	Imatinib	Nilotinib	Dasatinib
Patients whole cohort (n = 109)	39 (35.8%)	49 (45.0%)	21 (19.2%)
CD26+LSCs at TKI discontinuation			
POSITIVE (range)	19/39 (48.7%) (0,0048–0,1184 cells/μL)	30/49 (61.2%) (0,0001–0,1039 cells/μL)	12/21 (57.1%) (0,0063–0,0695 cells/μL)
NEGATIVE	20 (51.3%)	19 (38.8%)	9 (42.9%)
BCR::ABL1 transcript at TKI discontinuation			
POSITIVE (range)	19/39 (48.7%) (0,001–0,0046 copies)	26/49 (53.1%) (0,00024–0,007 copies)	10/21 (47.6%) (0,001–0,029 copies)
NEGATIVE	20/39 (51.3%)	23/49 (46.9%)	11/21 (52.4%)

Molecular response

BCR::ABL1 breakpoint mRNA monitoring has been assessed locally at each molecular laboratory of the centers participating in the study, by quantitative polymerase chain reaction (qPCR) methods, according to European Leukemia Net (ELN) guidelines (Hochhaus et al., 2020). Molecular response, expressed on an International Scale (IS), has been evaluated at the time of TKI discontinuation and at the subsequent time points (1, 2, 3, 6, 12, 18, and 24 months), until TFR loss, if any.

Statistical analysis

Descriptive statistics was carried out; qualitative variables were summarized by absolute frequencies and percentages, and the

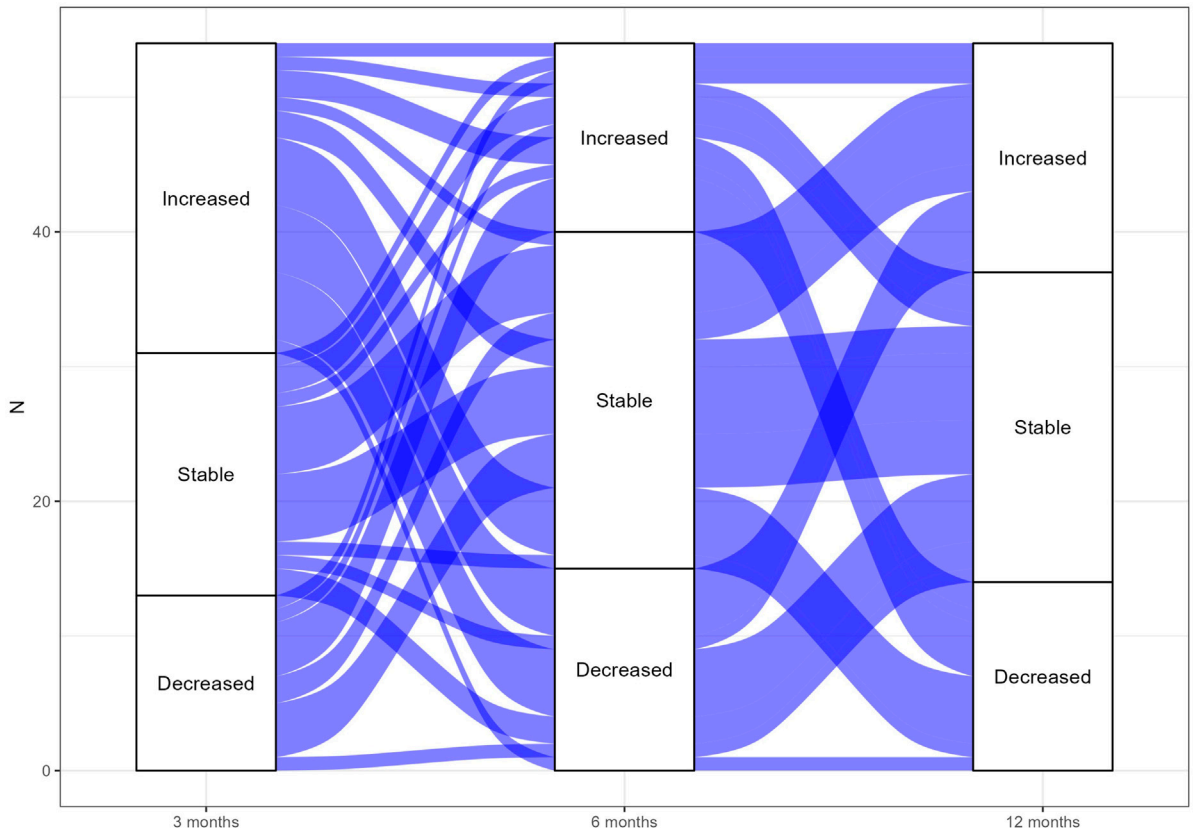

FIGURE 2
Alluvial plot of CD26+LSCs fluctuations for TFR-sustained patients. The alluvial plot represents CD26+LSCs fluctuations in TFR-sustained patients between 3, 6, and 12 months from TKI stop. The width of the lines corresponds to the number of patients who shared the same behavior.

quantitative ones were summarized with median and interquartile range. BCR::ABL1 and CD26+LSCs differences between TFR-sustained and TFR-loss were evaluated with the Mann–Whitney test, and the association between MR or treatment and response was evaluated with the Fisher exact test. The *post hoc* test for the association between treatment and TFR loss was evaluated by multiple Fisher exact tests with Bonferroni correction. Kaplan–Meier curves were performed to evaluate the survival without molecular relapse between patients double positive (i.e., positive for BCR::ABL1 copies and PB residual CD26+LSCs), double negative (i.e., negative for BCR::ABL1 copies and PB residual CD26+LSCs), and discordant at baseline (i.e., one positive and one negative test). A log-rank test was performed to compare the three different groups. A $p < 0.05$ was considered statistically significant. Statistical analyses were carried out with R version 4.0.1.

Results

Patient cohort clinical data

Between June 2017 and June 2022, 109 consecutive CP-CML patients at the time of TKI discontinuation were enrolled in the study. Clinical and biological data for CML diagnosis are reported in Table 1. In brief, 62/109 (57%) patients were males, and 47/109 (43%) were females; the median age at diagnosis was 53 years (range 19–76 years). The Sokal score was high in 16/109 (14.5%) patients, intermediate in 38/109 (35%), low in 49/109 (45%), and not available in 6/109 (5.5%), while the EUTOS score was high in 15/109 patients (14%), intermediate in 6/109 (5.5%), low in 82/109 (75%), and unknown in 6/109 (5.5%). The most frequent BCR::ABL1 transcript was b3a2 in 54/109 (49.6%) patients. Additional cytogenetic abnormalities were reported only in 3 patients (2.8%). First-line TKI treatment included imatinib in 39/109 (35.8%) patients, nilotinib in 49/109 (45%), and dasatinib in 21/109 (19.2%). The complete cytogenetic response was achieved at a median time of 3 months (range 2–21 months). Regarding MR after 12 months from TKI start, 56/109 (51.4%) patients had MR ≤ 3, 16/109 (14.7%) had MR 4, 14/109 (12.8%) had MR 4.5, and 16/109 (14.7%) had MR 5. The median TKI treatment duration before TKI withdrawal was 7 years (range 3–18 years). According to TKIs, the median treatment duration before TFR was 8 years (range 4–18 years) for patients on imatinib, 7 years (range 3–17 years) for patients on nilotinib, and 8 years (range 3–15 years) for those on dasatinib. After a median observation time of 33 months (range 2–63 months) from TKI discontinuation, a total of 38/109 (35%) patients failed TFR after a median time of 4 months (range 1–39 months). According to TKI treatment, these 38 TFR failing patients were distributed as follows: 19/39 (48.7%), 11/49 (22.4%), and 8/21 (38.1%) for patients

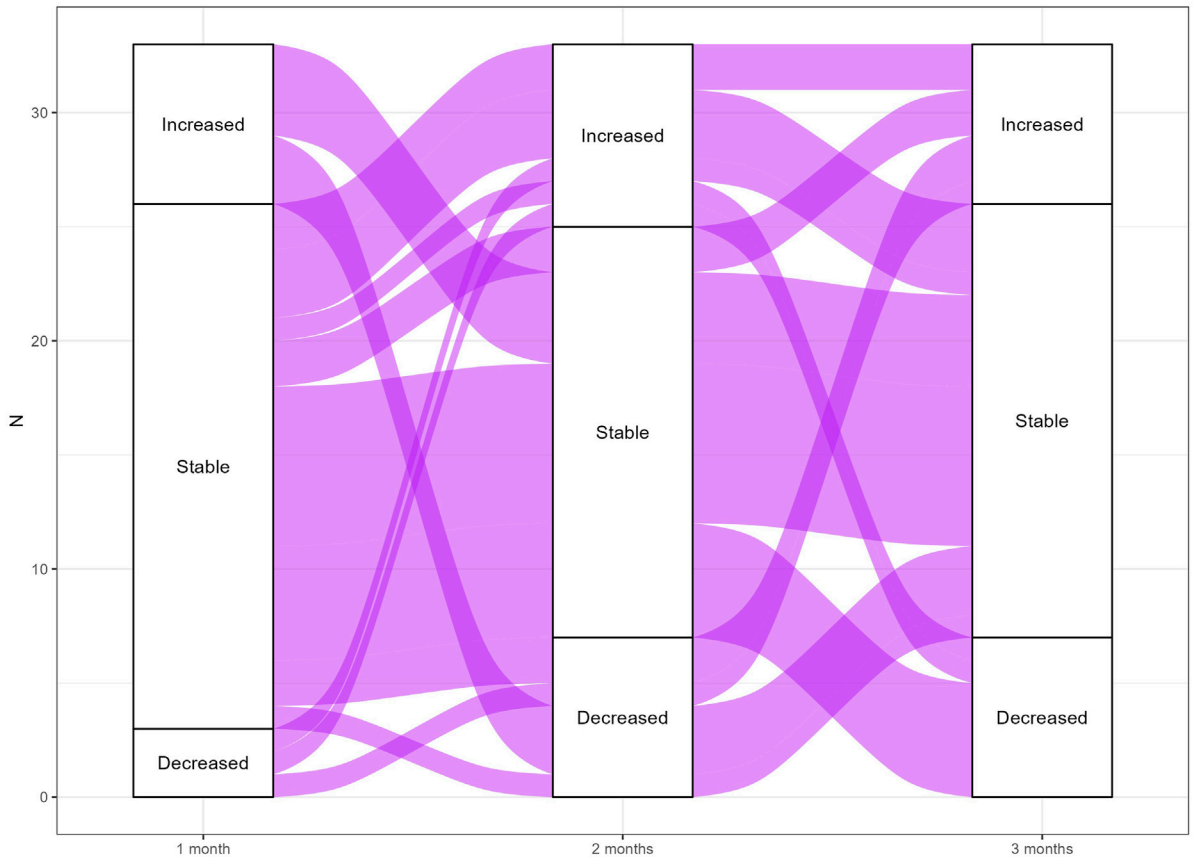

FIGURE 3
Alluvial plot of CD26+LSCs fluctuations for TFR-loss patients. The alluvial plot represents CD26+LSCs fluctuations in TFR-loss patients between 1, 2, and 3 months from TKI stop. The width of the lines corresponds to the number of patients who shared the same behavior.

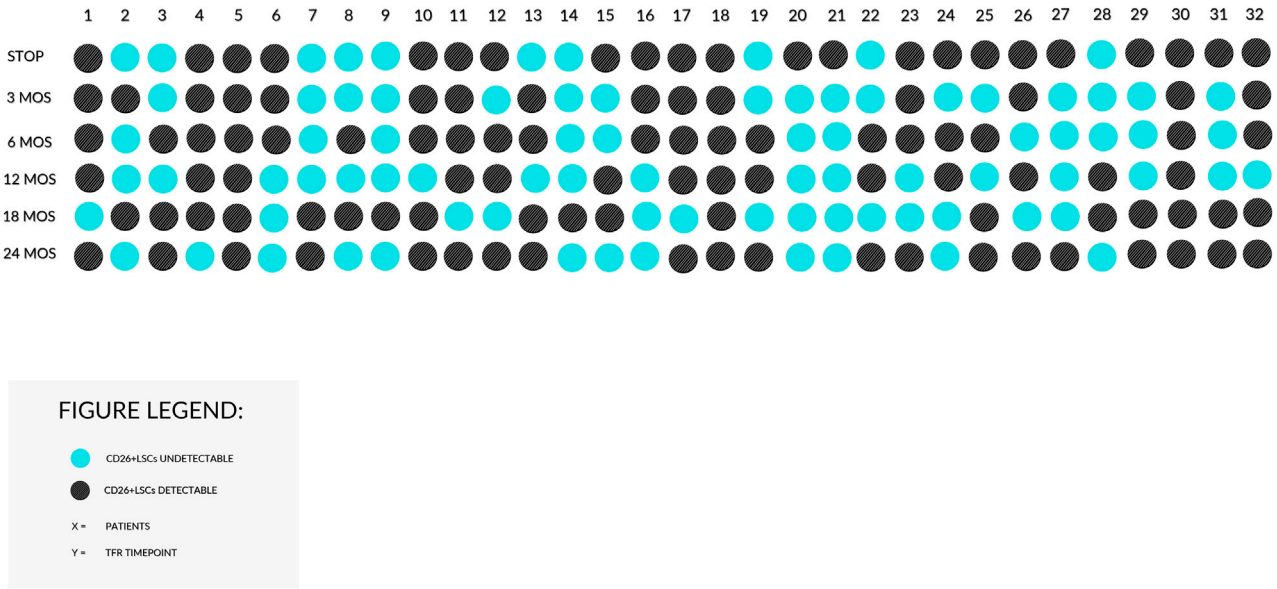

FIGURE 4
Behavior of CD26+LSCs in 32 TFR patients achieving 24 months of follow-up. The black dots stand for detectable PB CD26+LSCs, and the blue dots stand for undetectable CD26+LSCs. The great variability of the behavior of LSCs among patients with sustained TFR is demonstrated.

previously treated with imatinib, nilotinib, and dasatinib, respectively.

CD26+LSC evaluation and BCR::ABL1 transcript at the time of TKI discontinuation

All 109 CP-CML patients were evaluated for PB CD26+LSCs and BCR::ABL1 transcript at the time of TKI discontinuation. In 48/109 (44%), PB CD26+LSCs were undetectable, while in 61/109 (56%), we detected a median of 0,007 $CD26^+$ cells/μL (range 0,0001–0,1184 cells/μL). Regarding molecular evaluation at the time of TKI withdrawal, in 57/109 (51%) patients, BCR::ABL1 transcript resulted undetectable, while 52/109 (49%) patients scored positive with a median value of 0,0021 copies (range 0,0002–0,038 copies); in terms of MR, an MR > 3 was documented in 106/109 (97%) patients. No statistically significant correlation between detectable/undetectable CD26+LSCs and the rate of TFR loss was found (p = 0.616). Similarly, when correlating BCR::ABL1 ratio or BCR::ABL1 log reduction (expressed as MR4, MR4.5, and MR5) at the time of TKI stop with the incidence of TFR loss, we did not find statistically significant associations (p = 0.962 and p = 0.275, respectively). Additionally, we also investigated the correlation between molecular relapse and any possible combinations of detectable/undetectable CD26+LSCs and detectable/undetectable BCR::ABL1 transcript without finding statistically significant results (p = 0.646). Experimental data are summarized in Table 2; Figure 1. Table 3 details CD26+LSCs and BCR::ABL1 status at the time of imatinib, nilotinib, and dasatinib discontinuation; again, no correlation between residual CD26+LSCs, molecular response, and TFR loss was found. However, the pre-withdrawal type of TKI treatment was associated with TFR loss, and nilotinib treatment was followed by a TFR loss rate that was statistically significantly lower compared to that of imatinib (p = 0.039) but not of dasatinib.

CD26+LSC evaluation during TFR

According to the study design, PB CD26+LSCs were prospectively evaluated during TFR at established time points with a total of 541 measurements. To explore the behavior of residual CD26+LCSs over time, we focused our data analysis on three time points (3, 6, and 12 months) of the 71/109 CML patients still in TFR at the last follow-up.

CD26+LSCs during TFR were undetectable and detectable, respectively, in 24/71 (34%) and 47/71 (66%) patients at 3 months, 27/71(38%) and 44/71 (62%) patients at 6 months, and 24/62 (39%) and 38/62 (61%) patients at 12 months. When detectable, the median values of PB CD26+LSCs were 0,0128, 0,01 and 0,0155 cells/μL at 3, 6, and 12 months, respectively.

Fluctuations of CD26+LSCs were very different between TFR patients and not predictive of TFR loss. To better depict CD26+LSC fluctuations observed during the TFR time, we realized two alluvial plots of TFR-sustained (Figure 2) and TFR-loss patients (Figure 3). Furthermore, in a restricted fraction of patients, we analyzed the persistence of residual CD26+LSCs at 18 and 24 months of discontinuation: 25/40 (62.5%) patients at 18 months and 20/32 (62.5%) patients at 24 months of stable TFR still showed detectable CD26+LSCs in their PB with a median number of 0,0096 and 0,0058 cells/μL, respectively. Figure 4 displays CD26+LSCs behavior during TFR in 32 patients that achieved 24 months of follow-up, and it confirms the great variability observed between patients.

Discussion

Nowadays, in the era of precision and personalized medicine, the achievement of a safe TFR in CML patients discontinuing TKIs is the focal point of hematologists caring for CML patients. Although in the past years, the optimization of TKI use has limited TKI resistance and prompted the achievement of a sustained DMR, a considerable fraction of CML patients attempting TFR soon experience a molecular relapse and need to restart TKI treatment to avoid disease reappearance. The persistence of residual quiescent LSCs post-TKI treatment has been considered responsible for TFR loss. However, the mechanisms involved in this process have not been investigated. To our knowledge, this is the first report that prospectively monitored the behavior of circulating CML-specific CD26+LSCs in CML patients during TFR, exploring their possible active role in inducing molecular relapse. Previously, we demonstrated in a cross-sectional study that a consistent number of CML patients undergoing TKI treatment still harbored measurable residual PB CD26+LSCs, even when displaying a prolonged DMR and during a stable TFR. However, prospective data regarding how residual LSCs would behave over time from TKI withdrawal were lacking. In the prospective multicenter study reported here, 109 CML patients achieving standard criteria for TFR attempt were closely monitored for the presence of residual PB CD26+LSCs together with any clinical or molecular evidence of disease relapse.

From the clinical point of view, in line with the literature, we documented that 35% of CML patients discontinuing TKI treatment failed TFR after a median time of 4 months. We also found that the TKI type of treatment appeared to be correlated to the probability of TFR loss: in particular, imatinib-treated patients showed a significantly higher relapse rate when compared to those treated with nilotinib but not to those with dasatinib.

From the biological point of view, at the time of TKI withdrawal, the majority of CML patients (56%) still harbored a low yet detectable amount of CD26+LSCs in their peripheral blood, without any significant difference according to previous TKI treatment. However, surprisingly, and somehow in contrast to what was expected, the persistence of PB CD26+LSCs at the time of TKI discontinuation did not correlate with the incidence of relapse, and no threshold of residual CD26+LSC number predictive of TFR loss was found. Similarly, no correlation was found between the molecular response (expressed in BCR::ABL1 ratio or expressed in MR) at the time of TFR with the incidence of molecular relapse. Even when we analyzed combined values of PB CD26+LSCs and BCR::ABL1 copies at the time of TKI discontinuation, no statistically significant difference in terms of relapse rate was found between patients resulting in "positive" for both CD26+LSCs and BCR::ABL1 persistence, "negative" (absence of both residual CD26+LSCs and BCR::ABL1 copies), or "discordant" (either way positive for CD26+LSCs or BCR::ABL1).

The second aim of our study was to prospectively investigate the fate of residual CD26+LSCs along TFR and explore their behavior, both in relapsing and non-relapsing CML patients. As reported by alluvial plots (Figure 2 and Figure 3) by measuring CD26+LSCs at several time points during the study core (12 months), we observed fluctuating values of residual LSCs with great variability within each patient and between patients both in the subgroup that lost TFR (Figure 2) and in the 71 CML patients in sustained TFR (Figure 3). The long-lasting persistence of PB CD26+LSCs and the fluctuation of their values was also confirmed in 32 CML patients with stable TFR, in which flow cytometry evaluation of residual LSCs was monitored for up to 24 months (Figure 4).

Taken together, our findings strongly suggest that the persistence of quiescent CD26+LSCs at the time of TKI withdrawal and during TFR does not necessarily result in overt disease recurrence. Nevertheless, all 38 CML patients failing TFR showed clearly detectable PB CD26+LSCs at the time of molecular disease recurrence. The latter raises two questions. Are CD26+LSCs really the expression of a staminal, quiescent fraction of CML? If yes, which factors, if any, control these cells or induce them to enter into proliferation and differentiation pathways, thus causing the reappearance of active disease? To try to answer the first question, we further characterized the phenotype of PB CD26+LSCs and documented the co-expression of specific antigens, such as CD90 and Ki67 (Pacelli et al., 2022). The former confirmed the real stemness property of these CML-specific cell reservoirs; however, the latter, being a marker of "proliferative capability," is somehow in contrast with the concept of "quiescence" of LSCs. It could then be hypothesized that CD26+LSCs may enter proliferation and differentiation status only under certain conditions, such as if they are able to escape a possible immune control.

Even in the pre-TKI era, like in the last two decades of successful molecularly targeted therapy, several authors focused on both the role of the immune system in concurring with CML control (Caocci et al., 2015; Ilander et al., 2017) and immunotherapeutic strategies to be possibly added to TKIs in order to aim at CML cure (Cayssials and Guilhot, 2017). It seems evident that the unraveling of the inmost features of the TKI-insensitive stem/progenitor cell population, as well as its interactions with the immune system, may introduce an alternative way to monitor the BCR::ABL1-based molecular response to identify CML patients who achieved a cure/immune control of the disease and may, therefore, safely stop TKI. Recently, the PD-1/PD-L1 signaling pathway was described as an adaptive mechanism of immune resistance enacted by tumor cells to evade immune response (Butte et al., 2008). It has been shown that leukemic cells harbor the PD-L1 antigen, although there is little or no information about CML LSCs (Mumprecht et al., 2009; Christiansson et al., 2013; Sehgal et al., 2015).

As such, we have also tried to characterize CML-specific LSCs regarding their possible interaction with the immune system. Preliminary data in 47 newly diagnosed CML patients showed that the co-expression of PD-L1 on their CD26+LSCs is variable: 22/47 (47%) patients scored negative while 25/47 (53%) scored positive, albeit with a quite variable percentage of expression (median 28.5% of $CD26^+$ cells, range 12%–82.8%) (Pacelli et al., 2022). These preliminary pieces of evidence are in favor of a possible diverse fate of residual CD26+LSCs in every single patient with regard to immune evasion and, consequently, disease recurrence.

In conclusion, here, we showed prospectively and in a consistent number of CML patients that CML-specific CD26+LSCs may persist at a very low level during TFR, nevertheless allowing patients to live TKI-free and without experiencing a molecular relapse. On the other hand, the possibility to fail TFR is possible even when no CD26+LSCs are detectable at the time of TKI discontinuation. Other factors, including a variable interaction with the host immune system, may be crucial to unravel the fate of these cells and, consequently, to understand if and how to try to eradicate them with alternative approaches. Additional studies exploring the host immune system and its interaction with residual CD26+LSCs in CML patients with very long stable TFR are ongoing.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Siena medical ethics committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MB and ASi designed and coordinated the study. PP, ASa, and ASi collected and analyzed data and co-wrote the manuscript. PP, ASi, EB, and DR performed flow cytometry assays and data analysis. ASi and CM performed molecular assays in Siena. AC performed statistical analysis. ASa, EA, VG, MC, MA, SG, AI, LL, FS, CF, MD, VS, and EC enrolled patients in the study and collected patients' samples and clinical data. DR developed and coordinated flow cytometry assays. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Modulating retinoid-X-receptor alpha (RXRA) expression sensitizes chronic myeloid leukemia cells to imatinib *in vitro* and reduces disease burden *in vivo*

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Introduction: The ligand-activated transcription factors, nuclear hormone receptors (NHRs), remain unexplored in hematological malignancies except for retinoic acid receptor alpha (*RARA*).

Methods: Here we profiled the expression of various NHRs and their coregulators in Chronic myeloid leukemia (CML) cell lines and identified a significant differential expression pattern between inherently imatinib mesylate (IM)-sensitive and resistant cell lines.

Results: Retinoid-X-receptor alpha (*RXRA*) was downregulated in CML cell lines inherently resistant to IM and in primary CML CD34$^+$ cells. Pre-treatment with clinically relevant RXRA ligands improved sensitivity to IM *in-vitro* in both CML cell lines and primary CML cells. This combination effectively reduced the viability and colony-forming capacity of CML CD34$^+$ cells *in-vitro*. *In-vivo,* this combination reduced leukemic burden and prolonged survival. Overexpression (OE) of *RXRA* inhibited proliferation and improved sensitivity to IM *in-vitro*. *In-vivo, RXRA* OE cells showed reduced engraftment of cells in the bone marrow, improved sensitivity to IM, and prolonged survival. Both *RXRA OE* and ligand treatment markedly reduced BCR::ABL1 downstream kinase activation, activating apoptotic cascades and improving sensitivity to IM. Importantly, RXRA OE also led to the disruption of the oxidative capacity of these cells.

Conclusion: Combining IM with clinically available RXRA ligands could form an alternative treatment strategy in CML patients with suboptimal response to IM.

KEYWORDS

chronic myeloid leukemia (CML), imatinib, RXRA, RXRA ligands, resistance

1 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by a reciprocal chromosomal translocation t(9;22) that generates the BCR::ABL1 fusion protein, a constitutively active tyrosine kinase essential for the pathogenesis of the disease. Molecular targeted therapy with tyrosine kinase inhibitors (TKI) such as imatinib mesylate (IM) has dramatically improved the outcome of this disease. However, a proportion of patients fail to achieve or retain molecular response with TKIs leading to disease progression.

The mechanism of TKI resistance in CML has been extensively studied and broadly classified as BCR::ABL1 dependent and independent. BCR::ABL1-dependent mechanisms include mutations in the BCR::ABL1 kinase domain and over expression of BCR::ABL1 or gene expression. BCR::ABL1 independent resistance mechansim include the persistance of leukemic stem cells, activation of multiple signaling pathways, and epigenetic events (Milojkovic and Apperley, 2009; Dohse et al., 2010; Patel et al., 2017; Loscocco et al., 2019). Mutations in the BCR::ABL kinase domain are seen in ~30% of CML patients with suboptimal responses to imatinib. Some patients fail multiple TKIs without having documented kinase domain mutations. Effectively targeting these TKI-insensitive cells by combination therapies that interfere with various signaling pathways has been attempted previously (Prost et al., 2015; Irvine et al., 2016; Wagle et al., 2016; Agarwal et al., 2017; Finch et al., 2017). Although combining repurposed clinical agents such as venetoclax (Ko et al., 2014; Carter et al., 2016), pioglitazone (Prost et al., 2015), and tigecycline (Kuntz et al., 2017) with TKIs are shown to eliminate leukemic stem cells (LSCs) selectively, these drugs are not translated into clinics for CML treatment. Targeting nuclear hormone receptors (NHRs) with specific ligands has improved disease outcomes and survival in solid tumors (Culig, 2014; Wong et al., 2014; de Bono et al., 2018; Rathkopf et al., 2018; Smith et al., 2018; Munster et al., 2019). However, except for the role of all-trans retinoic acid (ATRA) in acute promyelocytic leukemia (APL) (Grignani et al., 1998; Lengfelder et al., 2005; Zheng et al., 2005; Park et al., 2008), no studies show the role of NHRs in modulating imatinib resistance in CML cells.

NHRs are a family of ligand-dependent transcription factors frequently dysregulated in various cancer types, rendering this family of proteins potential therapeutic targets for cancers (Petrie et al., 2007; Feldman et al., 2014; Quintero Barceinas et al., 2015). NHR ligands bind to the ligand binding domain of the receptor, which dissociates the co-repressors from the receptor and facilitates the binding of the co-activator for receptor activation (Sonoda et al., 2008). NHR ligand treatments improved disease outcomes with limited toxicity in various solid tumors. The most widely explored NHRs are estrogen (ER) and androgen receptor (AR), and their ligands are used as monotherapy or combination therapy for breast and prostate cancer. Long-term treatment of ER ligand tamoxifen in patients with breast cancer reduced disease mortality and death compared to no tamoxifen therapy (Group EBCTCG EBCTC, 2011; Davies et al., 2013). AR ligands (Flutamide, Bicalutamide, Enzalutamide, and Apalutamide) increased metastasis-free survival in patients with metastatic prostate cancer (Culig, 2014; Wong et al., 2014; de Bono et al., 2018; Rathkopf et al., 2018; Smith et al., 2018). Glucocorticoid receptor (GR) ligand, Relacorilant in combination with Nab-paclitaxel improved outcomes with limited toxicity in patients with adenocarcinoma and ovarian cancer (Munster et al., 2019). Retinoid-X-receptor alpha (*RXRA*) ligand bexarotene in combination with chemotherapy was more effective associated with better survival, and improved response in refractory cutaneous T-cell lymphoma (CTCL) (Duvic et al., 2001a; Duvic et al., 2001b; Pileri et al., 2013), metastatic breast cancer (Esteva et al., 2003; Yen et al., 2004; Yen and Lamph, 2005) and non-small cell lung carcinoma (NSCLC) (Khuri et al., 2001; Edelman et al., 2005). The role of NHRs in myeloid malignancies is relatively less explored except for the use of RARA (retinoic acid receptor alpha) ligand all-trans retinoic acid (ATRA) as a differentiation agent in APL. Preliminary results from our laboratory suggest that NHRs such as *PPARG* and *RXRA* were downregulated in chemoresistant acute myeloid leukemia (AML) cell lines (Karathedath et al., 2013), suggesting that NHRs can be modulated to improve chemosensitivity.

There are limited reports on the use of RXRA ligands in hematological malignancies. Rexinoid-X-receptors (RXR) are members of the NHR superfamily (Evans and Mangelsdorf, 2014). 9-cis retinoic acid, 9 cis-13,14-Dihydroretinoic acid, and long-chain fatty acids are natural ligands for RXR (Arnold et al., 2012; Jones et al., 2015; Rühl et al., 2015; Niu et al., 2017). In mammalian cells, three genes code for three RXR isoforms- *RXRA*, *RXRB*, and *RXRG* (Nohara et al., 2009). *RXRs* either homodimerize or heterodimerize with other NHRs for transcriptional activation. The heterodimeric partners of *RXRs* include retinoic-acid receptor (*RAR*), vitamin-D-receptor (*VDR*), peroxisome proliferator-activated receptors (*PPAR*), liver-X-receptor (*LXR*), pregnane-X-receptor (*PXR*), and the constitutive androsterone receptor (*CAR*) (Bugge et al., 1992; Kliewer et al., 1992; Willson and Kliewer, 2002; Calkin and Tontonoz, 2012). *RARA* and *RXRA* expression is downregulated in AML cells compared to myelodysplastic syndrome (Welch et al., 2014). Several studies have shown the efficacy of bexarotene as a pan-RXR ligand in AML. Bexarotene treatment induced differentiation and apoptosis in AML blast cells *in-vitro* and in AML cell lines (Altucci et al., 2005; Sanchez et al., 2014). AML patients treated with bexarotene showed differentiation *in-vivo* (Tsai et al., 2008). Bexarotene, combined with decitabine, was well tolerated and showed a modest response in older relapsed AML patients (Welch et al., 2014). The dual activation of RXRA-RARA heterodimers by the specific ligands, ATRA and bexarotene effectively targeted MLL-AF9 myelomonocytic leukemic cells *in-vitro* and improved the survival *in-vivo* in mice models (Di Martino et al., 2021a). DT448/9PP mutated *RXRA* receptor overexpression enhanced transcriptional activity leading to differentiation and loss of colony-forming ability in KMT2A-MLLT3 transformed AML cells (Di Martino et al., 2021b). Bexarotene treatment increased the protein expression of RXR isoforms (*RXRA*, *RXRB* & *RXRG*) in SH-SY5Y cells (Dheer et al., 2018). Bexarotene treatment increased the protein expression of *RXR* and *PPARG* in brain cells (Zuo et al., 2019). RXRA ligand treatment increased the mRNA expression of other NHRs, such as *PPARD*, *LXRA*, & *LXRB* (Di Martino et al., 2021a), in MLL-AF9 AML mice *in-vivo*.

Few studies have addressed the role of NHRs in modulating TKI sensitivity in CML. PPARG agonist pioglitazone was reported to target CML cells effectively by down-regulating transcription factors such as

STAT5, CITED2, and HIF2a, which are overexpressed in CML stem cells (Prost et al., 2015). PPARG ligand-thiazolidinedione has been reported to synergize with TKIs and target CML stem cells (Prost et al., 2015; Glodkowska-Mrowka et al., 2016). CML patients treated with pioglitazone combined with imatinib achieved a deeper molecular response of MR4.5 at 12 months (Rousselot et al., 2017). However, the role of other NHRs and the effect of their ligands in overcoming inherent TKI resistance in CML is still not completely understood.

We investigated the differential expression profile of NHRs in CML cell lines that are inherently imatinib sensitive vs. resistant. We also assessed the effect of RXRA ligand treatment combined with imatinib on improving TKI sensitivity in CML cell lines, primary cells *in-vitro,* and in a CML cell line-derived xenograft mice model.

2 Materials and methods

2.1 Chemicals and reagents

Imatinib Mesylate (SML1027), methyl thiazolyldiphenyl-tetrazolium bromide (MTT), 9-cis retinoic acid (9cRA), Bexarotene (Bexa), acitretin (ACI), SR11237, all trans retinoic acid (ATRA), testosterone, 17β-estradiol (17βE), pioglitazone (PIO), rosiglitazone (rosi), Triiodothyronine (T3) and GSK4716 were from Sigma-Aldrich (St Louis, MO, United States). Rabbit monoclonal p-CRKL (3,181), p-STAT5 (9,351), p-AKT (4,060), caspase-3 (9,661), PARP (5,625), and RXRA (3,085) were from Cell Signaling Technologies (Danvers, MA, United States). β-actin (A5441) was from Sigma-Aldrich (St. Louis, MO), and horseradish peroxidase-conjugated goat anti-rabbit IgG (1: 10,000), goat anti-mouse IgG (1:10,000) from Cell Signaling Technologies (Danvers, MA, United States). Imatinib Mesylate used for mice model experiments is a gift from NATCO, India.

2.2 Cell lines and primary cells

CML cell lines JURL-MK1, KCL22, Lama84, KYO1, EM2, and KU812, were a kind gift from Dr. Markus Muschen, University of California San Diego (UCSD), with approval from DSMZ, Germany. Cell lines were cultured in RPMI medium with 10%-FBS and antibiotics (100 U/ml-penicillin and 100 μg/mL-streptomycin) in a humidified atmosphere with 5% CO_2 at 37°C.

Bone marrow or peripheral blood samples were collected after obtaining written informed consent from imatinib naïve adult CP-CML patients at diagnosis before the initiation of therapy. This study was approved by the Institutional Review Board of Christian Medical College, Vellore (IRB Min No. 8704). Primary CML cells from blood or bone marrow were processed and enriched for CML $CD34^+$ cells, as reported previously (Rajamani et al., 2020).

2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from CML cell lines (JURL-MK1, KU812, KYO1, EM2, Lama84, and KCL22) and treated with or without NHR ligands using Trizol reagent. RNA (1 μg) was converted to cDNA using random hexamer and reverse transcriptase enzyme (High-capacity cDNA synthesis kit, Thermo Scientific).

2.4 NHR profiling

To identify the basal expression of NHRs and coregulators in CML cell lines, we used an NHR RT^2 PCR array (SA Biosciences, Germany) (https://geneglobe.qiagen.com/product-groups/rt2-profiler-pcr-arrays). The expression of each NHR was normalized using five housekeeping genes (*ACTB*, *GAPDH*, *B2M*, *HGPRT* & *RPLP0*), as recommended by the manufacturers. The data was analyzed using SA biosciences web-based analysis software (www.sabiosciences.com/pcr/arrayanalysis.php).

2.5 q-RT-PCR analysis of NHR expression

The expression of *AHR* (TaqMan assay ID: Hs00169233_m1), *AR* (Hs00171172_m1), *ESR1* (Hs01046816_m1), *ESRRG* (Hs00976243_m1), *PPARG* (Hs01115513_m1), *RXRA* (Hs0107640_m1), *RXRB* (Hs 00232774_m1) and *THRA* (Hs00268470_m1) were evaluated by qRT-PCR. Imatinib influx transporter *SLC22A1/hOCT1* (HS00427554-m1) expression was measured using TaqMan assay after treating the primary CML cells with NHR ligands. *GAPDH* was used as the housekeeping gene for these targets. Fold change in the expression of other NHRs such as *RARA*, *PPARG,* and *VDR* was measured by RQ-PCR using SYBR green chemistry (SYBR premix Ex TaqII (Tli RNAseH Plus), Takara, Bio, Shiga, Japan), and *ACTB* was used as the housekeeping gene to calculate the relative expression (ΔΔCt) of these targets.

2.6 In-vitro-cytotoxicity assay

CML cell lines JURL-MK1, KCL22, Lama84, KYO1, EM2, and KU812 [1×10^5], were treated in a 96-well plate with increasing concentrations of imatinib (0–500 nM) for 48 h. Cell viability was measured at standard wavelength 570 nm and reference wavelength 630 nm using EL800 microplate reader with KC junior software (Biotek Winooski, VT, United States).

CML cell lines (KCL22 & Lama84) were pretreated with NHR agonists (agonists and their concentrations as listed in Supplementary Table S1) for 24 h, followed by treatment with increasing concentration of imatinib (0 nM–500 nM). All the experiments were performed in triplicates. The inhibitory concentration of 50% (IC-50) was calculated using Adapt software.

As suggested by previous reports (Austin et al., 2015; Wang et al., 2017), bulk primary CML cells [3×10^5] were pretreated with NHR agonists for 24 h, followed by treatment with increasing concentration (0–100 uM) of imatinib for 48 hrs.

2.7 RNA expression by nanostring

RNA was extracted from CML $CD34^+$ cells ($n = 39$) and healthy donor $CD34^+$ cells ($n = 9$) using total RNA purification plus kit (Norgen Biotek Corp, Canada). Expression of *RXRA* was assessed using nCounter, and data counts were normalized with housekeeping genes–*ACTB*, *GAPDH* & *GUS* using nSolver software (NanoString, Technologies, Seattle, WA).

2.8 Colony forming unit assay

Primary CML (bulk & $CD34^+$) or healthy donors (peripheral blood mononuclear cells—PBMNCs & $CD34^+$) cells (2×10^5) were treated with and without RXRA ligands 9-cis retinoic acid, acitretin, or bexarotene in combination with imatinib for 24 h; 1×10^4 cells were seeded in methylcellulose medium (MethoCult™ H3334 classic, StemCell Technologies, Vancouver, Canada). Colonies were visualized using a phase-contrast microscope (×10 objective) and enumerated after 14 days.

2.9 Apoptosis assay

Primary CML $CD34^+$ cells (2×10^5) were treated with RXR ligands 9-cis retinoic acid, acitretin, or bexarotene for 24 h, followed by imatinib for 48 h. Cells were washed with 1XPBS and stained with Annexin-V conjugated with allophycocyanin (APC) and 7-Aminoactinomycin D (7AAD) (BD-bioscience, San Diego, CA). BD-Accuri C6 (BD Biosciences, Franklin Lakes, NJ) with BD Accuri C6 software (Version 1.0.264.21) was used for flow cytometry analysis.

2.10 Western blot analysis for BCR-ABL downstream signaling pathway

CML cell lines (KCL22, Lama84) or primary CML cells were treated with RXRA ligands (9-cis retinoic acid, acitretin, or bexarotene) for 24 h, followed by imatinib for 48 h. The whole-cell lysate was prepared using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease-phosphatase inhibitor cocktail (Roche, IN, United States) and 2 mM phenyl methyl sulfonyl fluoride (Sigma Aldrich). Total protein concentration was measured using the Bradford assay. 50 ug of protein was resolved in a 10% SDS PAGE. Anti-p-CRKL, anti-p-AKT, anti-p-STAT5, anti-caspase3, and anti-PARP antibodies were used, and β-actin was used as the loading control.

2.11 Overexpression of RXRA in CML cell line

The RXRA plasmid containing the full-length cDNA, pLX304 (clone ID- HsCD00437134), was purchased from the DNASU plasmid repository (Arizona, United States). Lentivirus particles were prepared by transfecting 293T cells with the RXRA cDNA plasmid and packaging plasmids. Lentiviruses were collected after 48, 60, and 72 h, pooled together, filtered, and concentrated using a lenti-X concentrator (Takara, Bio, Shiga, Japan), and stored at −80°C until use. Lama84 cells were transduced with lentiviruses in the presence of polybrene, followed by spinfection. RXRA OE virus transduced Lama84 cells were selected by blasticidin (1 mg/mL).

2.12 CML cell line-derived xenograft (CDX) mice model

293T cells were transfected with plasmid lentiX-Luc2 containing a luciferase reporter for lentivirus production. KCL22 cells were transduced with lentivirus containing the luciferase reporter. Luciferase transduced KCL22 ($1*10^6$ cells) were then transplanted into sub-lethally irradiated (2.5Gy) 8–10 weeks old NSG mice (Jackson Laboratory, United States) via tail-vein. Disease development was monitored by measuring luciferase expression by injecting D-luciferin substrate and imaging using an IVIS Spectrum *in-vivo* imaging system (PerkinElmer, United States). One week after transplantation, the mice were treated orally with either imatinib mesylate (100 mg/kg dissolved in water) (NATCO-Pharma Limited, India) or acitretin (10 mg/kg dissolved in corn oil) or in combination for 21 days. Engraftment of KCL22 cells in the mice was assessed by measuring luciferase expression every week for 6–7 weeks. All animal experiments were carried out per protocols approved by the Institutional Animal Care and Use Committee (IAEC No. 5/2019).

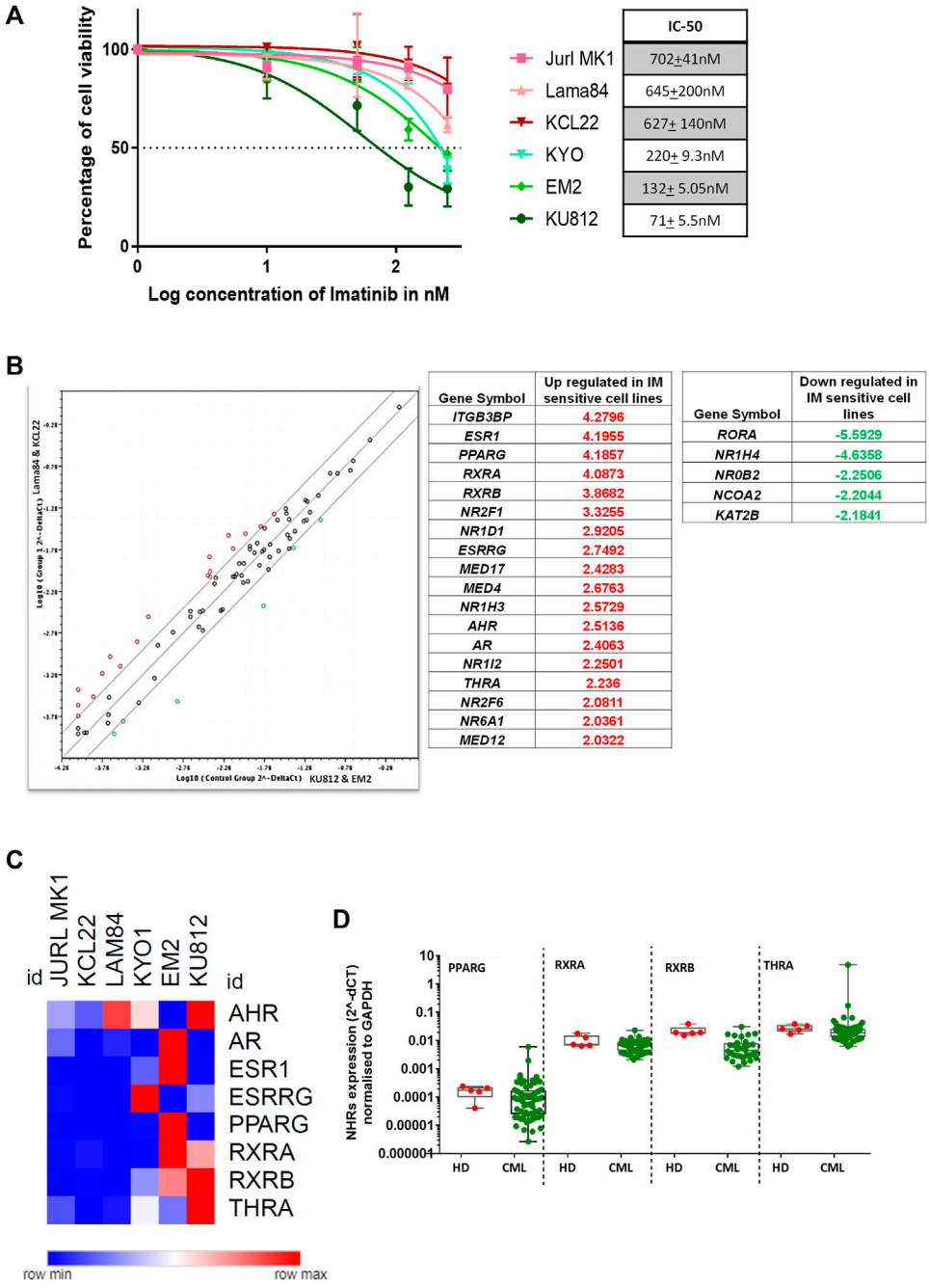
RXRA/empty vector transduced Lama84 ($2*10^6$) cells were transplanted into sub-lethally irradiated (2.5Gy) 8–10 weeks old NSG mice via tail-vein. The leukemic burden in peripheral blood was assessed using flow cytometry by measuring human CD45 expression (BD-bioscience, CA). The percentage of survival of mice was evaluated by Kaplan-Meir survival analysis.

2.13 Extracellular flux analysis using seahorse XFe24 analyzer

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse-XFe24 Analyzer (Agilent Technologies). Briefly, Lama84 EV and RXRA OE 4×10^5cells per well (4 replicates) were seeded in a 24-well XF24 plate coated with retronectin (Takara Bio, Shiga, Japan). Thirty minutes before analysis, the medium was replaced with Seahorse XF media (Agilent Technologies, Santa Clara, CA, United States), and the plate was incubated at 37°C. Three sequential measurements of OCR and ECAR were taken at basal, proton leak (oligomycin), maximal respiration (FCCP), and OXPHOS inhibition (rotenone and Antimycin-A) to assess bioenergetics.

2.14 Statistical analysis

Paired *t*-test, non-parametric *t*-test, and ANOVA with Kruskal-Wallis's correction were used where appropriate. All statistical analysis were done using GraphPad Prism-v5 software, and a p-value < 0.05 was considered statistically significant.



Gene Symbol	Up regulated in IM sensitive cell lines
ITGB3BP	4.2796
ESR1	4.1955
PPARG	4.1857
RXRA	4.0873
RXRB	3.8682
NR2F1	3.3255
NR1D1	2.9205
ESRRG	2.7492
MED17	2.4283
MED4	2.6763
NR1H3	2.5729
AHR	2.5136
AR	2.4063
NR1I2	2.2501
THRA	2.236
NR2F6	2.0811
NR6A1	2.0361
MED12	2.0322

Gene Symbol	Down regulated in IM sensitive cell lines
RORA	-5.5929
NR1H4	-4.6358
NR0B2	-2.2506
NCOA2	-2.2044
KAT2B	-2.1841

FIGURE 1
Nuclear hormone receptors and coregulators show differential expression in imatinib sensitive vs. resistant CML cell lines **(A)** Percentage cell viability in CML cell lines KU812, Lama84, KYO-1, JURL-MK1, EM2, and KLC22, treated with imatinib for 48 h; Imatinib IC-50 for each cell line is depicted (right). **(B)** Table showing upregulated and downregulated NHRs in imatinib sensitive (Control group—KU812, EM2) compared to resistant (Group-1—KCL22, Lama84) CML cell lines based on the RT^2 NHR profiling (SA Biosciences) results. **(C)** Validation of profile results by qRT-PCR in CML cell lines classified as resistant (JURL-MK1, KCL22, and Lama84) and sensitive (KYO-1, EM2, and KU812) based on imatinib IC-50. The expression of each NHR normalized to *GAPDH*. **(D)** Expression of selected NHRs in granulocytes from healthy donors (n = 5) and primary CML bulk cells (n = 69).

3 Results

3.1 Differential expression profile of NHRs in imatinib sensitive vs. resistant CML cell lines

We performed an *in-vitro* cytotoxicity assay to identify CML cell lines that are sensitive or resistant to IM. KU812, EM2, and KYO1 cell lines were identified as sensitive, and JURL-MK-1, KCL22, and Lama84 cell lines as resistant to IM. The kill curves and IC-50 of the cell lines tested are listed (Figure 1A). To identify whether the NHR expression differs between the IM sensitive (EM2, KU812) and resistant (KCL22 and Lama84) cell lines, we performed NHR profiling using SA Biosciences PCR-based array. There was differential expression (keeping a cut-off ≤ or >2 folds) of 9 NHRs

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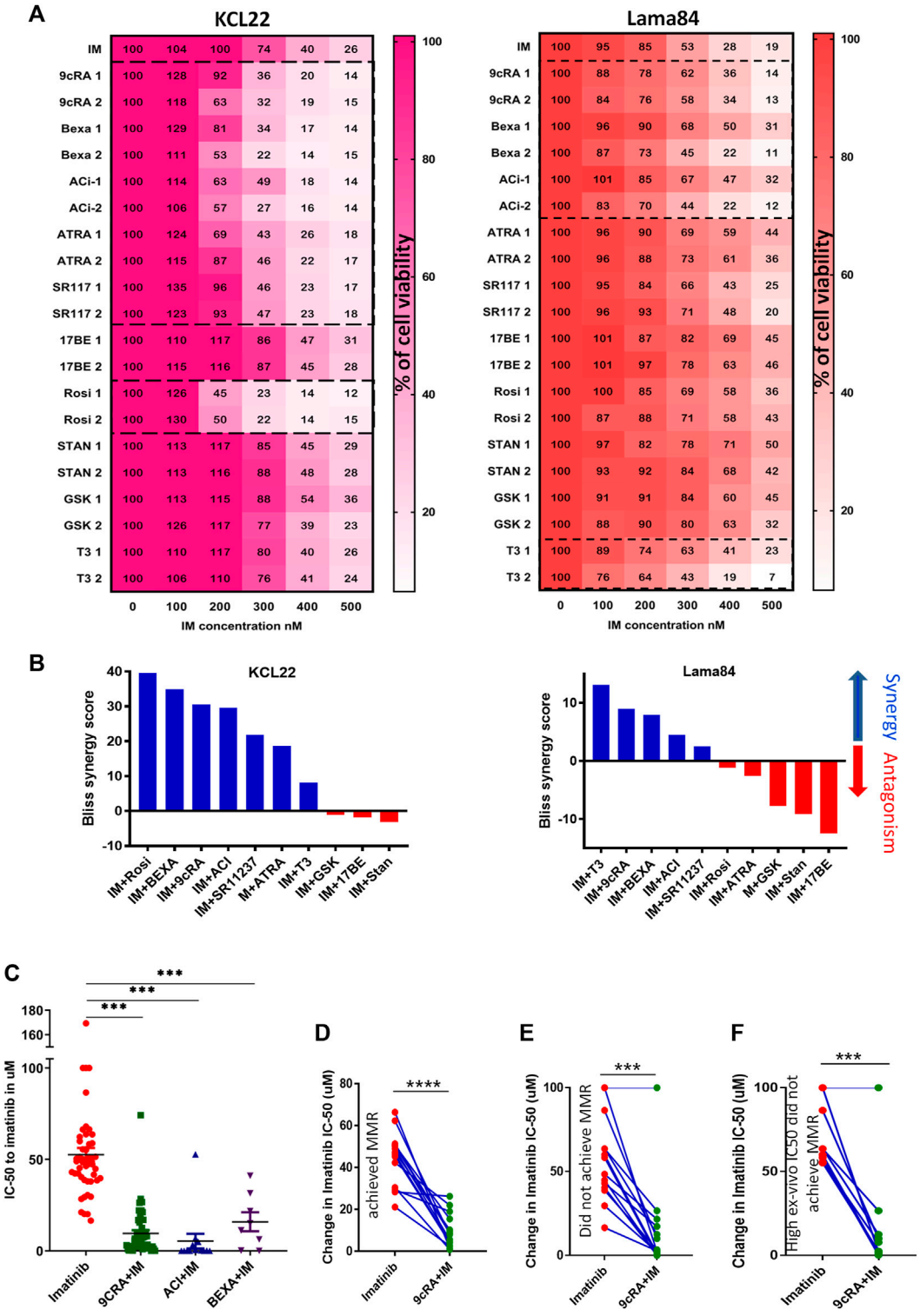

FIGURE 2
RXRA ligand treatment significantly reduced IC-50 to IM in CML cell lines and primary CML cells **(A)** Representative heat maps showing the cell viability measured at 48 h in CML cell lines KCL22 and Lama84 treated with ligands specific to downregulated NHRs in combination with imatinib. The *X*-axis shows the increasing concentrations of imatinib (100–500 nM), and the *y*-axis shows different NHR ligands (two different concentrations). **(B)** Bliss synergy score were analysed in cell lines treated with NHR ligands in combination with imatinib using synergy finder. The higher the synergy scores, the higher the synergy, and the lower the synergy scores, the antagonism. **(C)** The difference in IC-50 between imatinib alone vs. in combination with ligand [9-cis-retinoic acid (n = 42), acitretin (n = 13), and bexarotene (n = 8)] was compared using paired t-test and the p-value calculated by Tukey's multiple comparison test. The change in imatinib IC-50 with and without 9-cRA in primary CML cells treated with 9-cRA from patients who achieved MMR at 12 months **(D)**, those who did not achieve MMR at 12 months **(E)**, and those who did not achieve MMR with high IC-50 to imatinib **(F)**. p-value was calculated by paired t-test. Significance values: ***$p < 0.001$.

and 13 coregulators between IM-sensitive and resistant cell lines (Figure 1B). Compared to resistant cell lines, 8 NHRs (*ESR1, PPARG, RXRA, RXRB, ESRRG, AHR, AR, THRA*) and ten coregulators (*ITGB3BP, NR2F1, NR1D1, MED17, MED4, NR1H3, NR1I2, NR2F6, NR6A1, MED12*) were upregulated and *RORA* & 4 coregulators (*NR1H4, NR0B2, NCOA2* & *KAT2B*) were downregulated in imatinib-sensitive cell lines. A Representative Heatmap illustrates the expression profile of NHRs and coregulators (Supplementary Figure S1). Further validation by q-RTPCR revealed NHRs such as *RXRA*, *RXRB,* and *THRA* to be upregulated in the IM-sensitive cell lines compared to IM-resistant cell lines. In contrast, all the other NHRs (*AR*, *ESR1*, *ESRRG*, *PPARG*) were upregulated in at least one of the IM-sensitive cell lines (Figure 1C). To check if the expression pattern of NHRs was unique to CML, we compared the expression of selected NHRs between healthy donor (HD) granulocytes and primary CML cells. There was no significant difference in the expression pattern (Figure 1D).

3.2 RXRA ligand treatment significantly reduced IC-50 to imatinib in CML cell lines and primary CML cells

We next attempted to see if treatment with ligands specific to the downregulated NHRs in the inherently IM-resistant cell lines (KCL22, Lama84) could improve IM sensitivity. The concentration of the ligands used for this treatment is listed (Supplementary Table S1). Treatment with RXRA ligands (9cRA, bexarotene, acitretin, ATRA, SR11237) and PPARG ligand rosiglitazone decreased cell viability in combination with IM compared to IM alone in the KCL22 cells (Figure 2A). In the Lama84 cells, RXRA ligands bexarotene, acitretin, 9cRA, and THRA ligand T3 decreased cell viability (Figure 2A). The combination index (CI) was calculated using the bliss synergy score mathematical model to test the synergy between the combination of ligand and IM. KCL22 cells treated with rosiglitazone, bexarotene, 9cRA, acitretin, SR11237, ATRA, and T3 showed high synergy with IM, whereas GSK4716, 17-β estradiol, and stanozolol showed antagonism with IM (Figure 2B). Ligands such as T3, 9cRA, bexarotene, acitretin, and SR11237 showed synergy, and rosiglitazone, ATRA, GSK4716, stanozolol and 17-β estradiol showed antagonism with IM in Lama84 cell line (Figure 2B).

Based on these results, the ligands that sensitized either cell line to IM were tested in the primary CML cells. Treatment with RXRA ligands (9cRA, acitretin, and bexarotene) improved IM IC-50 *in-vitro* in primary bulk cells obtained from imatinib naive CML patients (Figure 2C). Other NHR ligands such as ATRA, 17-β-estradiol, pioglitazone, and T3 did not improve IC-50 to IM (Supplementary Figures S2A–E). Based on the patients' molecular response status at 12 months, the primary CML samples were grouped as attained major molecular response (MMR) or no MMR. 9cRA significantly decreased IM IC-50 *in-vitro* in samples from patients who achieved in all samples irrespective of the molecular response status (Figures 2D, E; Supplementary Figure S3). Primary CML cells from patients who did not achieve MMR and with high IC50 to IM (above median IC-50-53uM) also were sensitized to IM with 9cRA combination treatment (Figure 2F).

3.3 RXRA ligand treatment decreased cell viability and colony-forming unit in primary CML CD34$^+$ cells

As IM treatment does not eliminate the CML LSCs, we next tested the effect of RXRA ligands alone and in combination with IM in purified primary CML CD34$^+$ cells *in-vitro*, and the cell viability and colony-forming capacity were assessed. RXRA ligands, either alone or combined with IM, significantly decreased cell viability (Figure 3A) and colony-forming capacity (Figures 3B, C). Interestingly, acitretin alone treated CML CD34$^+$ cells showed a significant reduction in cell viability compared to imatinib alone, 9cRA alone, or Bexa alone or in combination (Figure 3A). Treatment of healthy PBMNCs with RXRA ligands 9cRA, bexarotene, and acitretin in combination with IM did not reduce cell viability compared to IM alone, suggesting that the cytotoxic effect of RXRA ligands in combination with imatinib is specific to CML cells (Supplementary Figures S4A–C). We then compared the expression of *RXRA* in CML CD34$^+$ and HD CD34$^+$ cells. *RXRA* expression was significantly lower in CML CD34$^+$ cells than in HD CD34$^+$ cells (Figure 3D). We also probed for *RXRA* expression across CML chronic phase (CP), accelerated phase (AP), and blast crisis (BC) patient samples from the GSE4170 dataset and found significantly reduced expression of *RXRA* in BC-CML samples compared to CP and AP (Figure 3E). These results suggest that either alone or combined with IM, RXRA ligands could selectively target CML CD34$^+$ cells.

3.4 RXRA ligand treatment inhibits BCR-ABL downstream signaling and activates the apoptotic cascade in CML cells in combination with imatinib

RXRA forms a homodimer or heterodimerizes with other NHRs, such as RARA, VDR, and PPARG to regulate cellular proliferation, differentiation, and apoptosis (Gilardi and Desvergne, 2014). To identify the expression of other NHRs post RXRA ligand treatment in CML primary cells, RNA expression of *RARA*, *VDR*, and *PPARG* was tested. RXRA ligands treatment resulted in consistent upregulation of *RARA* and *VDR* and downregulation of *PPARG* (Supplementary Figure S5). RXRA ligand treatment also increased RXRA protein levels in the CML cell lines (Figure 4A).

We then checked the effect of these RXRA ligands on the BCR-ABL signaling pathways (p-CRKL as a marker of proliferation, p-AKT as a marker of survival, and p-STAT5 as a marker of quiescence) in CML cell lines and primary cells. RXRA ligands decreased p-CRKL, p-AKT, and p-STAT5 at the protein level in combination with IM (Figure 4B) in CML cell lines. Primary CML cells treated with RXRA ligands combined with IM also showed inhibition of downstream signaling pathway p-CRKL (Figure 4C).

Next, we examined the effect of these RXRA ligands on CML cells in inducing apoptosis. CML cell lines treated with RXRA

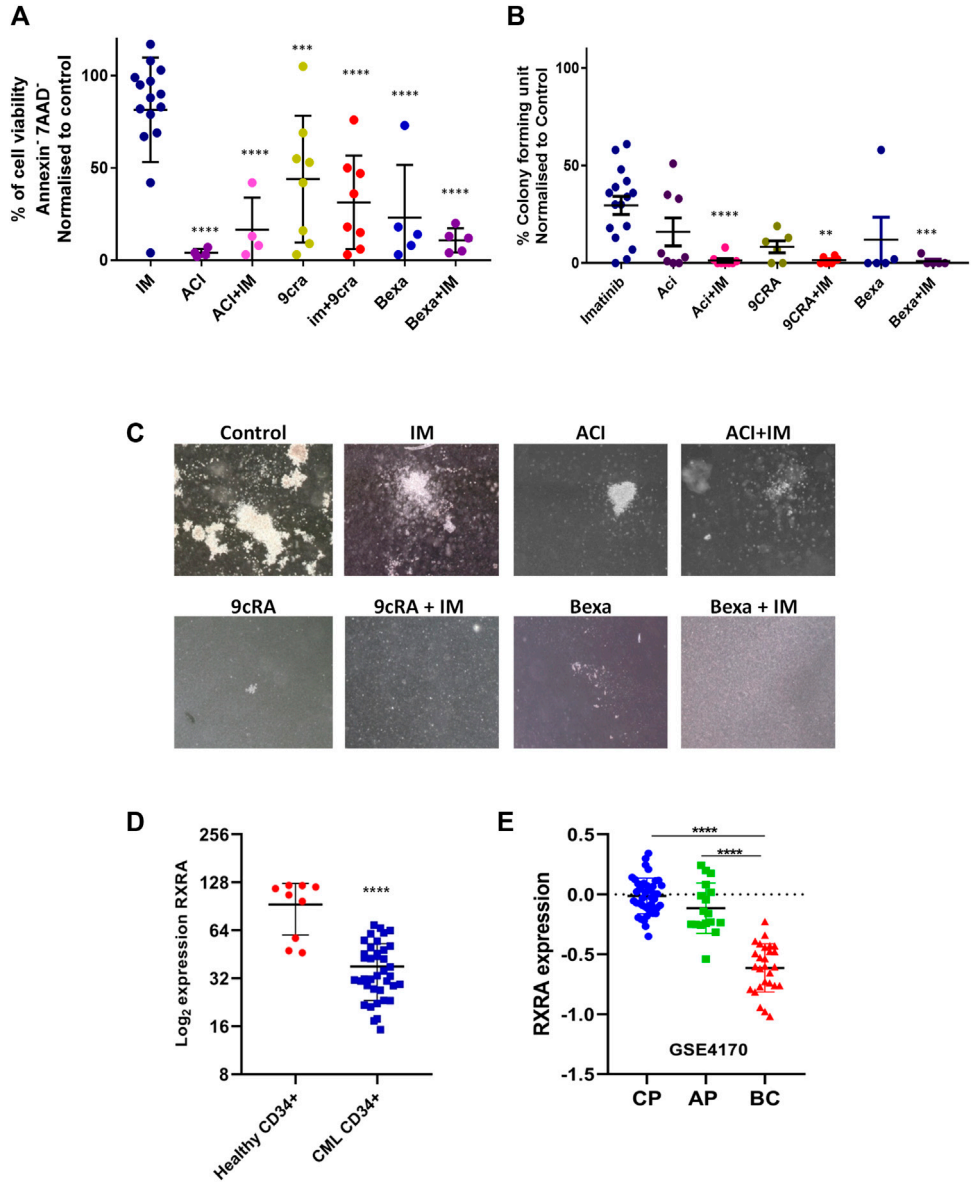

FIGURE 3
RXRA ligands in combination with imatinib decreases cell viability and colony-forming unit in primary CML CD34$^+$ cells **(A)** Percentage cell viability in primary CML CD34$^+$ cells treated with rexinoids (Aci n = 4, 9CRA n = 8 & Bexa n = 5) in combination with imatinib. Significance was calculated compared to imatinib-treated cells. p-value was calculated by Tukey's multiple comparison test. **(B)** Colony-forming capacity in primary CML CD34$^+$ cells treated with rexinoids (Aci n = 8, 9CRA n = 6 & Bexa n = 5) combined with imatinib; colonies were scored on day 14. Significance was calculated compared to imatinib-treated cells. p-value was calculated by Tukey's multiple comparison test. **(C)** Representative images of the colony-forming units in primary CML CD34$^+$ cells treated with imatinib alone, rexinoids alone, and in combination. **(D)** *RXRA* mRNA expression in healthy donor CD34$^+$ cells (n = 9) and CML CD34$^+$ (n = 39) cells analyzed using Nanostring nCounter. *RXRA* expression was normalized to *ACTB, GAPDH & GUSB* and presented in Log2. **(E)** RXRA expression values from the GSE4170 dataset in CML chronic phase (CP), accelerated phase (AP), and blast crisis (BC) patients. p-value was calculated by the Mann-Whitney U test. Significance values: $^{**}p < 0.01$; $^{***}p < 0.001$.

ligands combined with IM showed a significant increase in cleaved caspase-3 and cleaved PARP expression (Figure 4D) and increased apoptosis combined with IM (Figure 4E) as well as with 2nd generation TKIs, dasatinib and nilotinib (Supplementary Figures S6A, B). These results indicate that RXRA ligands improve IM sensitivity by decreasing the BCR-ABL downstream signaling pathways and increasing apoptosis in CML primary cells and cell lines.

3.5 Molecular overexpression of *RXRA* in Imatinib resistant Lama84 cell line decreased proliferation, BCR-ABL downstream signaling, and OXPHOS resulting in improved imatinib sensitivity

As proof of principle, we evaluated if molecular overexpression of *RXRA* in the CML cell line would result in

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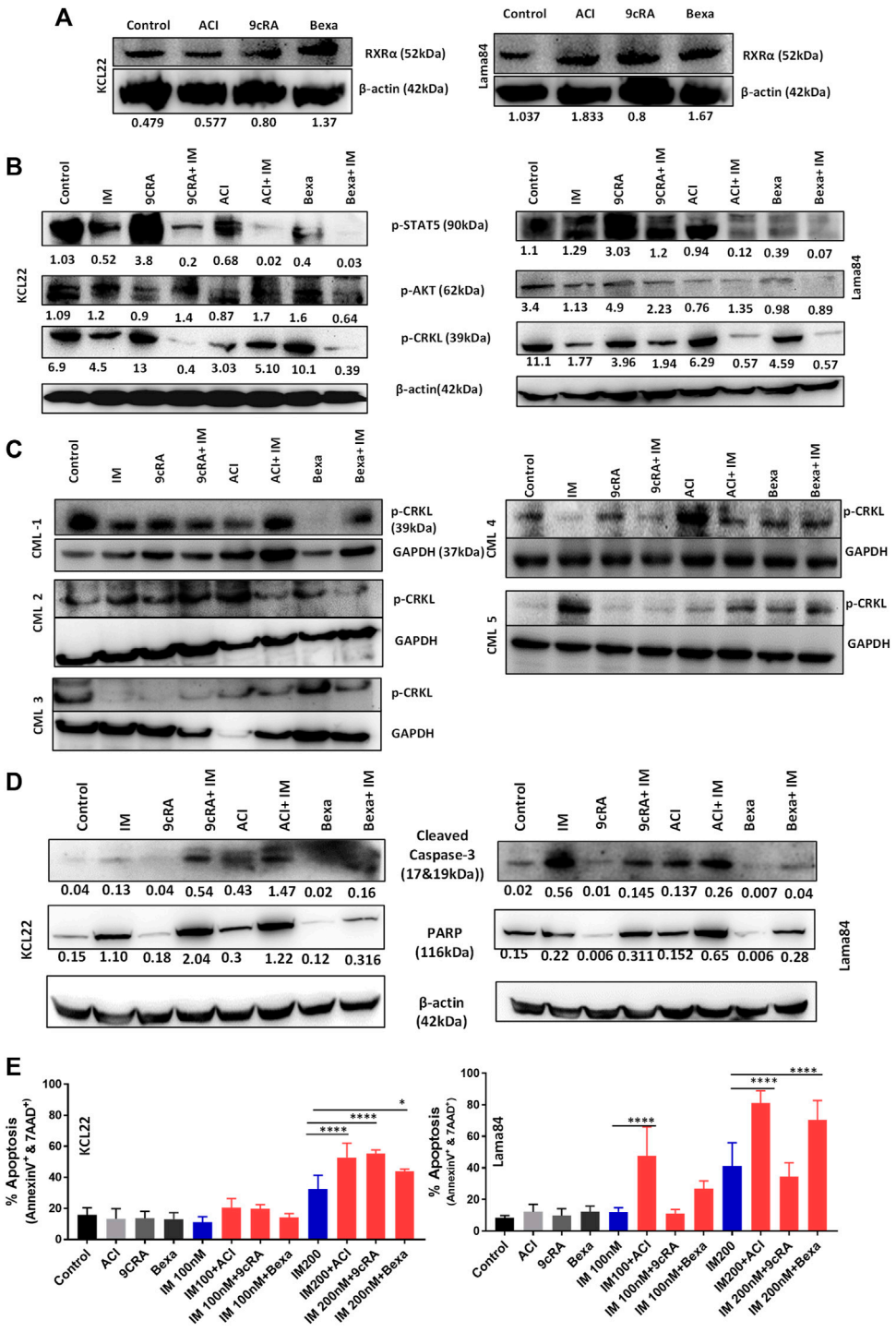

FIGURE 4
RXRA ligands decrease BCR-ABL signaling and activate the apoptosis pathway in CML cell lines **(A)** Western blot image showing increased RXRA protein expression in Lama84 and KCL22 cell lines treated with RXRA ligands Aci, 9CRA, and Bexa. β-actin was used as the loading control. **(B)** Western blot image showing the expression of BCR-ABL downstream signaling pathway proteins p-CRKL, p-AKT & p-STAT5 in KCL22, Lama84 cell lines, and **(C)** primary CML cells (n = 5) treated with rexinoids for 24 h, followed by imatinib treatment. β-actin/GAPDH was used as a loading control. **(D)** Western blot image showing the expression of apoptotic proteins of cleaved caspase-3 & PARP in CML cell lines treated with rexinoids followed by imatinib; β-actin was used as a loading control. **(E)** The percentage of apoptosis (Annexin-V and 7AAD positive cells) in CML cell lines treated with rexinoids with or without imatinib. p-value calculated by Tukey's multiple comparison test.

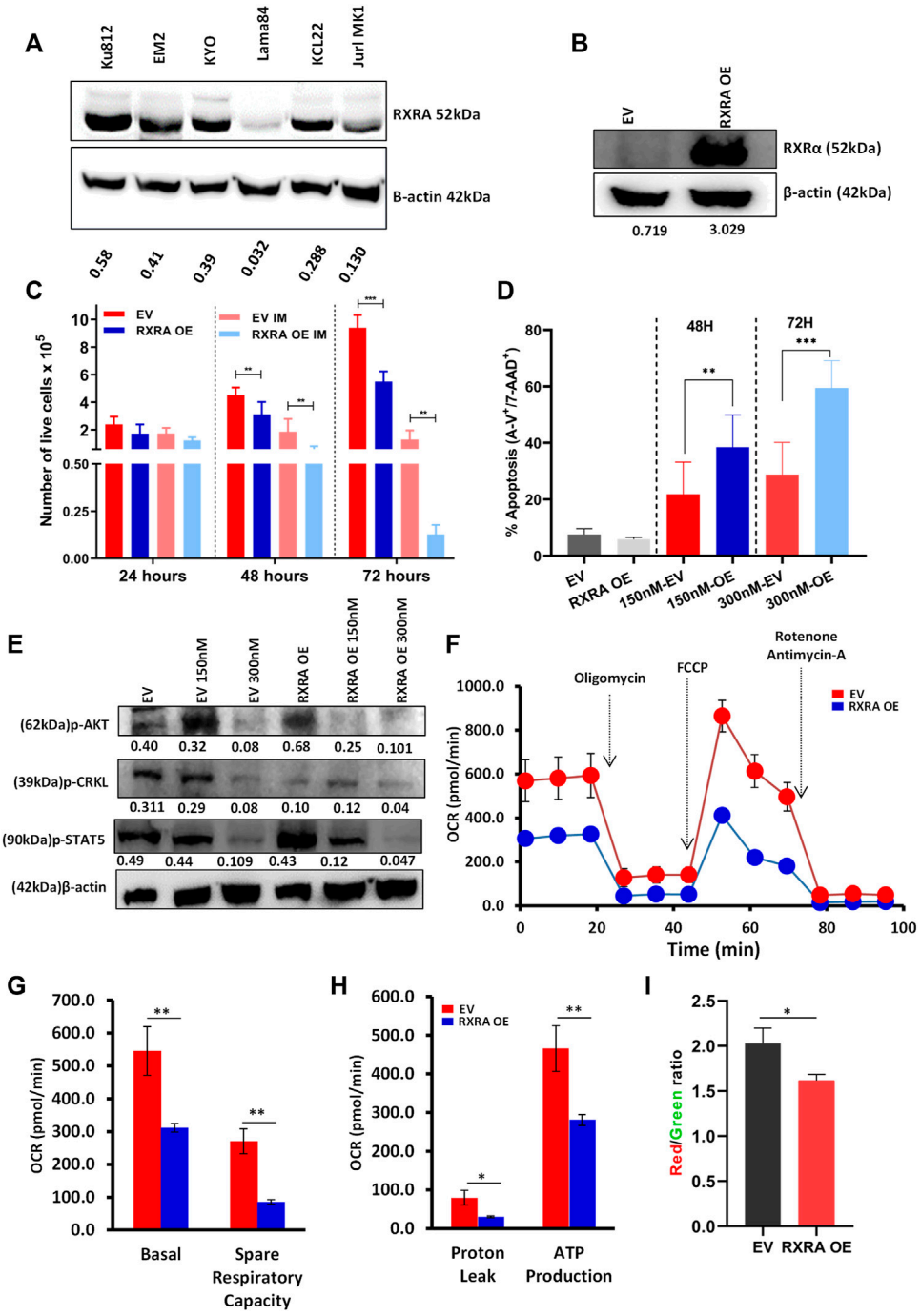

FIGURE 5
Molecular overexpression of RXRA improves imatinib sensitivity in the Lama84 cell line by inhibiting BCR-ABL signaling and oxidative phosphorylation **(A)** Basal RXRA expression in whole-cell lysate from CML cell lines KU812, EM2, Lama84, and KCL22 analyzed by western blot. Lama84 cell line with relatively low RXRA protein expression was transduced with RXRA OE plasmid, and overexpression was confirmed at the protein levels by western blotting **(B)**. The number of viable cells in Lama84 RXRA OE vs. EV with and without imatinib treatment was assessed using trypan blue exclusion assay (n = 3) at three different time points. The doubling time was calculated using exponential curve analysis, and the p-value was calculated using the Mann-Whitney U test **(C)**. EV and RXRA OE Lama84 cells were treated with 150 nM concentration of imatinib (n = 3), and the percentage of apoptosis was assessed by apoptosis assay (Annexin-V and 7AAD positive cells) at two different time points **(D)**. The p-value was calculated by Tukey's multiple comparison test. Western blot image showing the expression of BCR-ABL downstream signaling proteins (p-CRKL, p-AKT, and p-STAT5) in RXRA OE cells and EV cells **(E)**. Mito Stress Test for EV and RXRA OE cells using Sea horse extracellular flux analyzer **(F)**. Quantitative assessment of Basal OCR rates, Spare respiratory capacity, proton leak, and ATP production **(G,H)** (N = 4). **(I)** The quantitavie measure of mitochondrial membrane potential was analysed using the ratio of JC-1 dimer (Red) by JC-1 monomer (Green).

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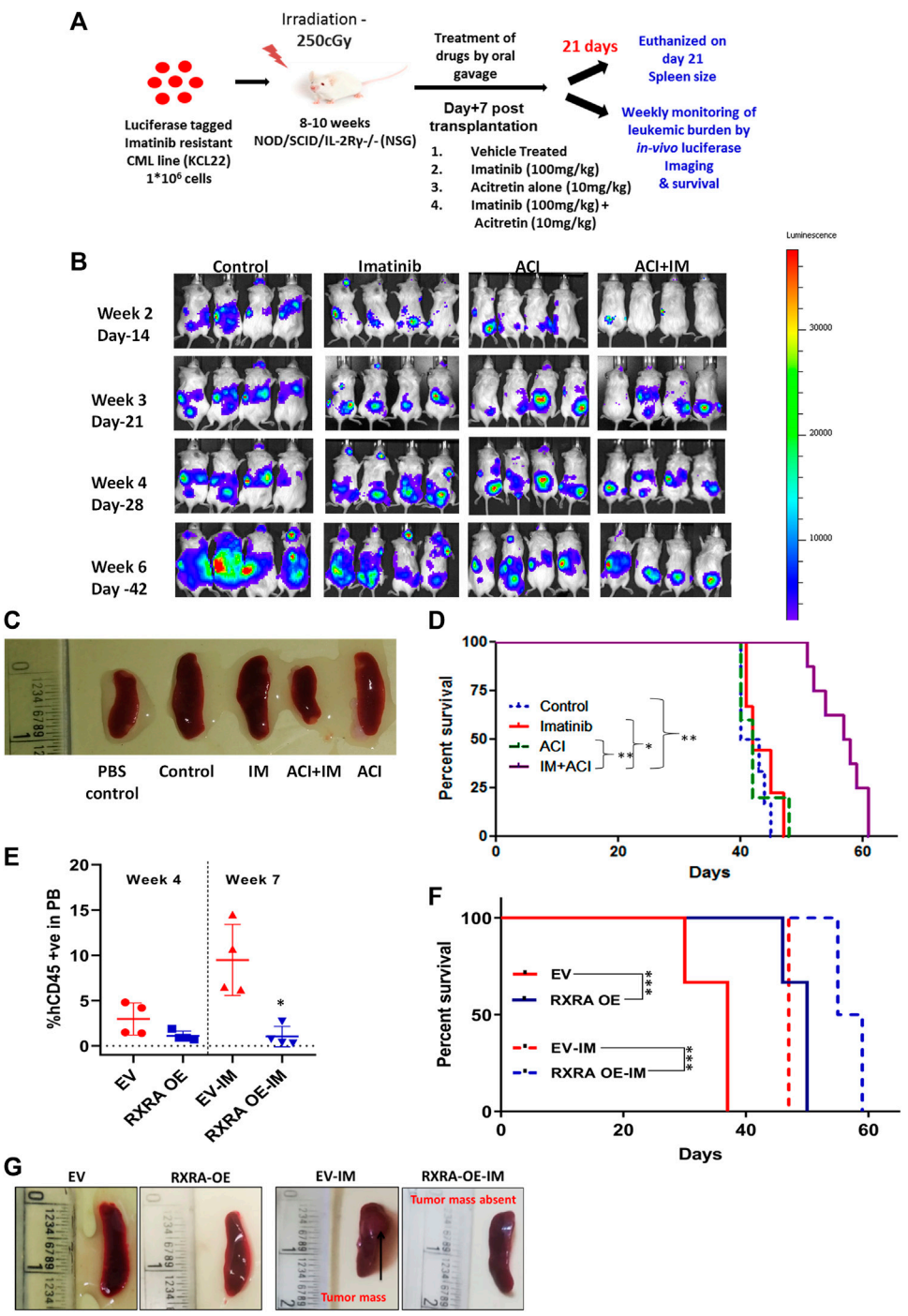

FIGURE 6
RXRA ligand acitretin in combination with imatinib decreases the leukemic burden and increases survival in the xenograft CML mice model **(A)** Outline of the CML cell line-derived (CDX) mouse model experiment. **(B)** Representative bioluminescence images showing *in-vivo* luciferase expression in CML mice treated with RXRA ligand with or without imatinib compared to the vehicle control mice. **(C)** Representative mice spleens in vehicle vs. treated animals. **(D)** Kaplan-Meier survival curve of NSG mice transplanted with KCL22 cell line and treated with IM alone (n = 9), ACI alone (n = 5), ACI + IM (n = 8), and vehicle control (n = 6). Percentage survival was calculated using the Kaplan-Meier analysis, and median survival was compared between treated groups vs. control groups by Dunn's multiple comparisons test. **(E)** Percentage of human CD45 cells from EV and RXRA OE injected mice after 4 weeks of transplantation and at week 7 between EV/RXRA OE treated with IM. **(F)** Kaplan-Meier survival curve of NSG mice transplanted with Lama84 EV (n = 3) and RXRA OE cell line (n = 4) (2*10^6 cells transplanted/mouse). Percentage survival was calculated using the Kaplan-Meier analysis, and median survival was compared between treated groups vs. control groups by Dunn's multiple comparisons test. **(G)** Representative spleens from mice injected with wild type vs. RXRA OE Lama84 cell line.

similar effects as that of ligand treatment. Overexpression (OE) of *RXRA* in the Lama84 cells with deficient basal protein expression (Figure 5A) of RXRA, resulted in significantly increased RXRA expression at the protein level (Figure 5B). Interestingly, RXRA OE alone considerably decreased the proliferative capacity of these cells and increased the sensitivity to imatinib in a time-dependent manner (Figure 5C; Supplementary Figure S7). We further validated the sensitivity to imatinib in RXRA OE cells by apoptosis and observed enhanced cytotoxicity to imatinib in these cells compared to the empty vector (EV) transduced (Figure 5D). We next assessed if the profound sensitivity to imatinib is due to inhibition of BCR-ABL downstream signaling pathways (p-CRKL, p-AKT & p-STAT5) as observed during ligand treatment. Immunoblotting of RXRA OE and EV cells revealed diminished p-CRKL signaling in the RXRA OE cells at the basal level. We observed complete inhibition of p-STAT5, p-CRKL, and p-AKT in a dose-dependent manner compared to EV, post-IM treatment in the RXRA OE cells (Figure 5E).

As leukemic cells rely on oxidative phosphorylation for energy demand and for withstanding TKI therapy, we assessed the bioenergetic profile of EV and RXRA OE cells. Intriguingly, RXRA OE markedly reduced the basal respiration rates indicative of restricted intrinsic ATP demand. RXRA OE significantly reduced ATP production, and the addition of mitochondrial uncoupler FCCP showed markedly reduced oxidative bursts compared to EV (Figures 5F–H). We further assessed the mitochondrial membrane potential (MMP) of EV and RXRA OE using JC-1 and found significantly reduced MMP in RXRA OE cells (Figure 5I). Interestingly, MMP measured post-RXRA ligand treatment in CML cell lines showed a significant decrease in MMP in the ACI-treated KCL22 cells, while there was no substantial change in Lama84 cells (Supplementary Figures S8A, B). These results collectively suggest that RXRA OE mimics the effect of RXRA ligand treatment in CML cells. Additionally, molecular and pharmacological activation of RXRA could potentially inhibit OXPHOS in CML cells improving sensitivity to IM.

3.6 RXRA ligand treatment and RXRA OE decreased leukemic burden and improved survival in cell-derived xenograft CML mice model by enhancing sensitivity to imatinib

As the *in-vitro* data suggested that RXRA ligands improved imatinib sensitivity and inhibited BCR-ABL downstream signaling in CML cell lines and primary CML cells, we investigated the *in-vivo* efficacy of acitretin combined with imatinib. A transplantable xenograft CML mouse model was developed by injecting luciferase-expressing KCL22 cells into sub-lethally irradiated NSG mice. Mice were treated with imatinib, acitretin, or a combination of both for 21 days (Figure 6A). Acitretin-treated mice showed ruffled fur coat and moderate weight loss. Intriguingly, mice treated with either imatinib alone or acitretin alone showed an increased incidence of hind leg paralysis compared to vehicle-treated mice and did not improve survival. While the combination of acitretin and IM treatment reduced spleen size, decreased leukemic burden, and significantly increased median survival (Figures 6B–D). *In-vitro* treatment of the HD PBMNCs and $CD34^+$ cells with acitretin in combination with imatinib did not significantly affect the viability of these cells as measured by apoptosis assay, suggesting that this effect is unique to CML cells (Supplementary Figures S9A, B).

Next, we evaluated the engraftment capacity of the RXRA OE and EV cells by transplanting an equal number of cells into sub-lethally irradiated NSG mice (Supplementary Figure S9C). RXRA OE hindered the engraftment capacity of the Lama84 cell line and improved survival. We also assessed the sensitivity to imatinib *in-vivo* in the RXRA OE/EV engrafted mice. Imatinib treatment for 21 days significantly reduced leukemic burden and prolonged survival in the RXRA OE cells transplanted mice compared to EV (Figures 6E, F; Supplementary Figure S9D). Imatinib treatment in EV-transplanted mice showed tumor mass formation but was absent in the RXRA OE mice treated with imatinib (Figure 6G). These comprehensive *in-vivo* results suggest that pharmacological and molecular approaches to activate RXRA could hinder leukemic cell engraftment, increase sensitivity to imatinib, and improve survival.

4 Discussion

Suboptimal response to TKI or TKI failure is associated with poor progression-free survival in patients with CML. Combining TKI with small molecules specific to pathways in which imatinib-resistant cells escape therapy could help eliminate the residual LSCs. In the present study, we observed differential expression of NHRs in imatinib sensitive vs. resistant CML cell lines when comprehensively screened for the expression of NHR and coregulators across CML cell lines. Upon treatment with ligands specific to the downregulated NHRs in the imatinib-resistant CML cell lines, only RXRA ligand treatment showed improved sensitivity to IM in both cell lines. Pioglitazone, a PPARG ligand, has been reported to eliminate CML LSCs but not the bulk primary CML cells (Prost et al., 2015; Wang et al., 2017). Combination treatment with Pioglitazone and imatinib induced complete molecular response (CMR) in patients who have not achieved CMR under long-term imatinib treatment in CML patients and remained in CMR after the withdrawal of imatinib (Prost et al., 2015).

Treating with ligands specific to the downregulated NHRs, including rosiglitazone (a PPARG ligand), showed a differential effect, sensitizing the KCL22 cell line but no impact on the Lama84 cell line in combination with IM. Similarly, THRA agonist T3 treatment sensitized Lama84 and did not affect KCl22. Although 17BE and stanozolol treatment improved viability in both cell lines, these ligands did not improve sensitivity to IM in bulk CML cells *ex-vivo*. As these ligands are present endogenously, it would be interesting to understand their effect on primary CML cells. Hence, we worked with RXRA ligands 9cRA, Aci, and bexarotene, which improved IM sensitivity in CML bulk and CML $CD34^+$ cells but had minimal effect on HD $CD34^+$ cells and PBMNCs. In addition, treatment with RXRA ligands

combined with imatinib decreased clonogenic potential in primary CML CD34$^+$ cells.

Prost et al. (2015) showed inhibition of p-STAT5 led to clearance of CML LSCs after treatment with *PPARG* agonist pioglitazone. When we assessed the BCR-ABL downstream signaling pathways, we observed that RXRA agonist in combination with IM resulted in decreased p-CRKL, p-STAT5, and p-AKT along with activation of the apoptotic cascade in CML cell lines and primary cells.

Lentiviral-mediated overexpression of *RXRA* in imatinib-resistant Lama84 cell line showed decreased proliferation, decreased BCR-ABL downstream signaling, and improved imatinib sensitivity *in-vitro*. Di Martino et al. (2021a) showed that deleting endogenous RXRA in an MLL-AF9 AML mouse model increased leukemogenic potential and reduced survival. The same authors subsequently reported that a constitutively overexpressing mutant variant of RXRA resulted in myeloid maturation and prolonged survival in the murine model (Di Martino et al., 2021a; Di Martino et al., 2021b). In line with this, in our RXRA overexpressing CDX CML mice model, we observed decreased leukemic burden and improved survival compared to EV-transduced CDX. We also found a significant reduction in *RXRA* expression in CML CD34$^+$ cells compared to HD CD34$^+$ and a reduction in the expression of *RXRA* in blast crisis CML patients compared to chronic phase, accelerated phase CML from the GSE4170 dataset indicating a potential role of *RXRA* in hindering leukemogenic capacity in CML.

It is reported that CML CD34$^+$ cells have a high oxidative capacity, which could be inhibited using tigecycline (Kuntz et al., 2017). Interestingly, we also identified that RXRA OE markedly inhibited the OXPHOS capacity of CML cells. Potentially RXRA ligands could mimic RXRA OE and have a disruptive effect on the oxidative potential of CML cells. In addition, RXRA OE and imatinib treatment significantly reduced leukemic cell growth *in-vivo* resulting in prolonged survival. Studies in solid tumors have demonstrated using RXRA agonists to increase sensitivity to chemotherapeutic drugs cisplatin and paclitaxel (Hermann et al., 2005; Blumenschein et al., 2008; Ramlau et al., 2008). Similar results were observed when the CML CDX model (KCL22 cell line) was treated with RXRA agonist acitretin combined with IM, improving survival. Interestingly, the agonist-treated arm had no survival benefit, unlike RXRA OE. The mechanisms of RXRA activation by pharmacological/molecular means may differ, and RXRA agonists (9cRA, ACI, and BEXA) may have a pleiotropic effect *in-vivo* compared to RXRA OE.

While our findings suggest the potential use of RXRA agonists in combination with IM in eliminating CML CD34$^+$ *in-vitro* and sensitizing CML bulk cells highly resistant to IM (IC50 > 53uM), a more robust *in-vivo* model to evaluate the therapeutic efficacy of this combination and anti-leukemogenic potential RXRA has on CML LSCs is needed. We are currently exploring the mechanism by which RXRA activation by both molecular/pharmacological means could orchestrate the downstream kinase activities.

We demonstrated differential NHR expression between imatinib-resistant and sensitive cell lines. The ligand-dependent activation of RXRA could be an effective CML target, affecting BCR-ABL1 downstream signaling and apoptotic pathway. Our *in-vitro* and *in-vivo* results suggest that using RXRA ligands could be an effective combination therapy to improve the imatinib response in patients with CML.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, CMC, Vellore (IAEC No. 5/2019).

Author contributions

BR and RI designed the research, performed experiments, analyzed results, and wrote the manuscript. EB, BB, DJ, SD, AP, RV, AM, and SK performed experiments and analyzed the results. AA and VM contributed to the analysis and review of the manuscript. SV designed the research, performed experiments, contributed to analysis and review of the manuscript. PB designed the research, performed experiments, analyzed results, wrote the manuscript, and procured funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1187066/full#supplementary-material

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Myelofibrosis at diagnosis is associated with the failure of treatment-free remission in CML patients

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The management of patients with chronic myeloid leukemia (CML) has been revolutionized by the introduction of tyrosine kinase inhibitors (TKIs), which induce deep molecular responses so that treatment can eventually be discontinued, leading to treatment-free remission (TFR) in a subset of patients. Unfortunately, leukemic stem cells (LSCs) often persist and a fraction of these can again expand in about half of patients that attempt TKI discontinuation. In this study, we show that presence of myelofibrosis (MF) at the time of diagnosis is a factor associating with TFR failure. Fibrotic transformation is governed by the action of several cytokines, and interestingly, some of them have also been described to support LSC persistence. At the cellular level, these could be produced by both malignant cells and by components of the bone marrow (BM) niche, including megakaryocytes (MKs) and mesenchymal stromal cells (MSCs). In our cohort of 57 patients, around 40% presented with MF at diagnosis and the number of blasts in the peripheral blood and BM was significantly elevated in patients with higher grade of MF. Employing a CML transgenic mouse model, we could observe higher levels of alpha-smooth muscle actin (α-SMA) in the BM when compared to control mice. Short-term treatment with the TKI nilotinib, efficiently reduced spleen weight and *BCR::ABL1* mRNA levels, while α-SMA expression was only partially reduced. Interestingly, the number of MKs was increased in the spleen of CML mice and elevated in both BM and spleen upon nilotinib treatment. Analysis of human CML-vs healthy donor (HD)-derived MSCs showed an altered expression of gene signatures reflecting fibrosis as well as hematopoietic support, thus suggesting MSCs as a potential player in these two processes. Finally, in our cohort, 12 patients qualified for TKI discontinuation, and here we observed that all patients who failed TFR had BM fibrosis at diagnosis, whereas this was only the case in 25% of patients with achieved TFR, further supporting the link between fibrosis and LSC persistence.

KEYWORDS
CML, TFR, fibrosis, leukemic stem cells, LSC, MSc

1 Introduction

Chronic myeloid leukemia (CML) is a prime example of the success of molecular-targeted therapy. The implementation of tyrosine kinase inhibitors (TKIs) that bind to and inhibit the oncogenic driver BCR::ABL1 has revolutionized treatment outcomes in this cancer entity. However, primary or secondary resistance, as well as leukemic stem cell (LSC) persistence, remain a problem that hinders the cure of this disease. The persistence of LSCs is clinically reflected by residual *BCR::ABL1*-expression in CML patients in major or deep molecular response (MMR, DMR), which often persist at a very low level despite long-term treatment. LSC persistence is a major obstacle for one of the main goals of CML therapy, i.e., treatment-free remission (TFR), where patients remain in MMR or DMR after discontinuation of TKI therapy. The first large clinical trial on TFR has shown that about half of CML patients relapse, usually within the first 6 months (Mahon et al., 2010). Further studies focused on additional aspects such as the specific TKI, course of initial response, duration or level of DMR prior to discontinuation of therapy with slight deviations in outcome [reviewed in (Cortes et al., 2019; Saglio and Gale, 2020; Osman and Deininger, 2021)]. Some predictive factors for success of TFR have been proposed, such as a longer period of DMR before discontinuation, older age, lower SOKAL risk score or longer time of TKI treatment before discontinuation [reviewed in (Atallah and Sweet, 2021)]. Based on the large number of studies, it is currently calculated that about two-thirds of newly diagnosed CML patients are eligible for TKI discontinuation, with half of them remaining in TFR.

A variety of mechanisms that could be responsible for the persistence of LSCs have been described. Looking at the microenvironment, we now know that the inflammatory signature of LSCs is driven not only by autocrine but also by paracrine mechanisms (Kuepper et al., 2019). Interestingly, some of the cytokines that have been shown to promote LSC persistence, such as interleukin-6 (IL-6) (Welner et al., 2015; Kuepper et al., 2019), interleukin-1β (IL-1β) (Zhang et al., 2016), tumor necrosis factor (TNF) (Herrmann et al., 2019; Agarwal et al., 2021) or transforming growth factor-beta (TGF-β) (Naka et al., 2010), have also been shown to support fibrotic processes in the bone marrow (BM).

BM fibrosis or myelofibrosis (MF) is a complex process that leads to the deposition of excessive extracellular matrix (ECM) within the BM, which, upon progression slowly replaces hematopoietic cells. The development of MF is potentially possible in all myeloid neoplasms (Sabattini et al., 2010) and is typically a slow and gradual process, where rapid changes of the ECM are rare. The origin of BM fibrosis is still poorly understood, but it is thought to involve the dysregulation of multiple cellular pathways, including cytokine signaling and inflammatory responses [reviewed in Gleitz and Benabid et al. (Gleitz et al., 2021)]. In the BM of fibrotic Philadelphia-negative (Ph^-) myeloproliferative neoplasms (MPNs), Gli^+ and $LepR1^+$ MSCs are driven into differentiation by a proinflammatory environment into a pro-fibrotic, myofibroblast-like cell type that then deposits excessive reticulin and collagen fibers (Ignotz et al., 1987; Lataillade et al., 2008; Decker et al., 2017; Schneider et al., 2017). The severity of BM fibrosis is often described with MF grades ranging from lower prefibrotic grade 1 to defined MF with grades 2–3 (Thiele et al., 2005; Swerdlow et al., 2017). In CML, the proportion of patients with MF varies widely from 30% to 100% of patients (Gralnick et al., 1971; Kvasnicka et al., 2001; Buesche et al., 2003; Hamid et al., 2019). Interestingly, imatinib seems to be able to either slow down or reverse MF (Beham-Schmid et al., 2002; Bueso-Ramos et al., 2004; Thiele et al., 2004; Buesche et al., 2007). An open question is whether the degree of MF at diagnosis has an impact on response to TKI treatment, with different studies showing conflicting results (Kantarjian et al., 2005; Buesche et al., 2007; Tanrikulu Simsek et al., 2016). Importantly, Buesche et al. (2007) showed that MF before start or within the first 3 months of imatinib therapy did not influence the probability of achieving a complete hematologic response during imatinib treatment. However, deeper responses such as complete cytogenetic remissions or MMR were less likely in patients with MF compared to patients without MF.

Given the supposed impact of MF on TKI response and the overlapping spectrum of cytokines that influence this process as well as LSC persistence, we analyzed here the relationship between BM fibrosis and TFR outcome in a small cohort of patients.

2 Materials and methods

2.1 Statistical analysis of patient data

Clinical data were collected from 57 patients from the RWTH Aachen University Hospital. All patients provided informed consent and analysis of human samples was approved by the local ethics board of the medical faculty RWTH Aachen (EK 206/09 and EK 391/20). MF grades were assessed by the local pathology department of the RWTH Aachen University Hospital for all patients except 3. Median age ± standard deviation of patients was 54.7 ± 15.4 years, 42 (74%) were male. Correlations between MF grade at diagnosis and TFR were assessed for patients undergoing discontinuation of the TKI after being at least 1.5 years in deep molecular remission (MR4 (Cross et al., 2012), *BCR::ABL1* <0.01%). Success of discontinuation was defined as stable MMR for at least 1 year without need for re-treatment. TFR failure was defined as loss of MMR. Optimal response (OR) at 12 months was achieved if the patient's *BCR::ABL1* levels were below 0.1% (MMR as defined in the International Randomized Study of Interferon and STI571 trial [IRIS] (Hughes et al., 2010)) 12 months after diagnosis and start of TKI treatment.

2.2 Animal experiments

FVB/N *SCLtTA/BCR::ABL1* (double transgenic, CML) mice and FVB/N *SCLtTA* (single transgenic, control) siblings were bred in-house and tetracycline was added to the drinking water (0.5 g/L, Sigma-Aldrich, St. Louis, MO, United States) in order to avoid the expression of *BCR::ABL1* (Institute for Laboratory Animal Science, Uniklinik RWTH Aachen). BM cells of control or CML mice were isolated from femur and tibia, separated by cell straining and 2×10^6

cells were transplanted into the wildtype 10 Gy irradiated FVB/N CD45.1$^+$ recipient mice by tail vain injection (Janvier Labs®). Mice were treated with cotrimoxazole (Ratiopharm, Ulm, Germany) for 2 weeks after transplantation. One week after transplantation, *BCR::ABL1* expression was induced by withdrawing tetracycline. After an additional week, treatment of CML mice with nilotinib (NIL, 50 mg/kg body weight) by daily gavage over 3 weeks was started. NIL (MedChemExpress, Monmouth Junction, NJ, United States) was resolved in 10% N-Methyl-2-pyrrolidon (Carl Roth) and 90% polyethylene glycol 300 (Sigma-Aldrich). Treatment controls were performed on both control and CML mice by administering the vehicle following the same procedure. All animal experiments were approved by the local authorities of North Rhine-Westphalia, Germany (LANUV Az. 84–02.04.2013.A072).

2.3 Immunohistochemistry (IHC) staining

Formalin preserved sternum specimen were embedded in paraffin and sectioned with a microtome and further processed following manufacturer instructions (Cell Signaling®). To perform the staining, sections were de-paraffinized and hydrated, followed by antigen retrieval with citrate buffer. Sections were then blocked by peroxidase followed by serum blocking. Sections were incubated overnight at 4°C with an anti-alpha-smooth muscle actin (α-SMA) primary antibody (add antibody number) at a 1:800 dilution. Next, slides were washed and incubated for 30 min with the secondary antibody (HRP, Rabbit #8114; Cell Signaling Technology®). Finally, sections were developed with diaminobenzidine, counterstained with hematoxylin and mounted with coverslips. Quantification of IHC staining was carried out using Fiji software (ImageJ®), after performing a color deconvolution (H DAB) (Crowe and Yue, 2023).

2.4 Fibrosis staining/grading of CML patients

After Formalin preservation, decalcification (EDTA) and paraffin embedding, 2.5–4 µm slides were produced by a microtome. For staining, a silver impregnation technique was used. The sections were de-paraffinized and hydrated and workflow was performed according to the following protocol: potassium permanganate (1%) for 5 min, oxalid acid (2%) for 5 min, iron alum for 5 min, silver solution ammoniacal for 10 s, formalin (5%) for 3 min, sodium thiosulphate (2%) for 2 min. Between each step, washing with distilled water was performed. Afterwards, nuclear fast red solution was used for 10 min for counterstaining. After immersing in alcohol (ascending, 70%–100%) and xylol, the slides were mounted with coverslips.

Grading occurred according to the WHO grading system (semiquantitative bone marrow fibrosis (MF) grading system proposed by WHO Classification of Tumours of Hematopoetic and Lymphoid Tissues (Swerdlow et al., 2017)). The four grades (MF-0 to MF-3) are defined as follows: MF-0: Scattered linear reticulin with no intersections, MF-1: Loose network of reticulin with many intersections, especially in perivascular areas, MF-2: Diffuse and dense increase in reticulin with extensive intersections, occasionally with focal bundles of thick fibers mostly consistent with collagen and/or associated with focal osteosclerosis, MF-3: Diffuse and dense increase of reticulin with extensive intersections and coarse bundles of thick fibers, consistent with collagen, usually associated with osteosclerosis. In heterogenous patterns, the final grade is determined by the highest grade present in at least 30% of the marrow area.

2.5 Flow cytometry analysis of murine bone marrow and spleen cells

BM and spleen cells were harvested and lysis of red blood cells was performed with a ammonium-chloride-potassium solution (AKC). Subsequently, cells were washed with PBS (Pan Biotech®) + 2% fetal bovine serum (FBS, PAN Biotech®) and then incubated for 20 min at 4°C with a 1:100 dilution with anti-CD41 conjugated with APC Cy7 fluorochrome (BioLegend®) in the dark. Next, cells were washed and resuspended in PBS + 2% FBS. In addition, FACS staining of B-cells (B220$^+$), granulocytes (GR-1$^+$/CD11b$^+$), T-cells (CD3$^+$), blasts (KIT$^+$), and erythrocytes (Ter119$^+$) was performed. Flow cytometer measurements were carried out using Gallios flow cytometer (Beckman Coulter®) and analyzed with Kaluza analysis software (Beckman Coulter®).

2.6 Isolation of MSCs

Human MSCs were obtained from BM aspirates of five CML patients at time of diagnosis and from hip bone donated after hip replacement surgeries of five healthy donors (HD). Cells were isolated by tissue culture plastic adherence after the mononuclear cell fraction was separated by Ficoll. Cells were seeded at 160,000 cells per cm^2 in Dulbecco's Modified Eagle Medium (DMEM) low glucose (Gibco®) supplemented with 10% of FBS, 1% each of L-glutamine, penicillin/streptomycin (both Gibco®) and cultured at 37°C with 5% CO_2. After 24 h, the medium was replaced and non-adherent cells removed by washing with phosphate-buffered saline (PBS, PAN Biotech®). Afterwards, adherent cells were maintained with medium replacement twice a week, detached with 0.25% trypsin-EDTA (Gibco®) after reaching confluence. MSCs were used for our experiments at passage 2 (p2).

2.7 Trilineage differentiation assays

MSCs were tested for their ability to differentiate *in vitro* into adipocytes, osteocytes and chondrocytes (Sudo et al., 2007). Briefly, in order to perform adipogenic differentiation, cells were cultured in medium consisting of DMEM high glucose (Gibco®), 10% FBS, 1 µM dexamethasone, 200 µM indomethacin, 500 µM 3-isobutyl-1-methylxantine and 10 µg/mL insulin (all Sigma®). On day 14–21, adipogenic cultures were washed with PBS, fixed in PFA 4% and stained with Oil Red O (Sigma®). For the osteogenic differentiation, cells were cultured in medium consisting of DMEM high glucose (Gibco®) supplemented with 10% of FBS (PAN Biotech®), 1% L-glutamine, 1% penicillin/streptomycin (both Gibco®), 10 nM dexamethasone, 10 mM β-glycerolphosphate and 50 µM ascorbic acid (Sigma®). After 21 days, cells were washed with PBS, fixed in PFA 4% and stained with Alizarin Red (Sigma®). For the chondrogenic differentiation, cells were cultured

in medium consisting of DMEM high glucose (Gibco®), 1% L-glutamine, 1% penicillin/streptomycin (both Gibco®), 100 nM dexamethasone, 0.17 mM L-Ascorbic acid-2-PO4, 100 µg/mL sodium pyruvate, Insulin Transferrin Selenium supplement (ITS, Sigma®) and 10 ng/mL TGF-β. On day 14, cultures were washed with PBS, fixed in PFA 4% and stained with Alcian blue (Sigma®). After visual inspection and image acquisition, quantification of the three stainings was performed using the following solvents: isopropanol for adipogenic staining, 10%-cetylpyridinium chloride solution for osteogenic staining and 6M Guanidine HCl solution for chondrogenic staining, the relative absorbance was measured with a plate reader (Multiskan FC, Thermo Fisher®).

2.8 MSC phenotype

MSC phenotype was assessed at passage 2 (p2) using the following monoclonal antibodies: anti-CD73-FITC (eBioscience®); anti-CD34-FITC (BD Biosciences®, BD); anti-CD45-PE (BD®); anti-CD90-PerCP-Cy5.5 (eBioscience®); anti-CD13-PE (eBioscience®); anti-CD11b -FITC (BD®) anti-CD44-APC and anti-HLA-DR-FITC (BD®). Staining of at least 50,000 cells was performed at 4°C for 15 min in the dark in presence of 1% human serum to avoid unspecific binding. Samples were acquired with a Gallios flow cytometer (Beckman Coulter®) and analyses were performed using FlowJo Software (BD®).

2.9 CFU-F assay

Log serial dilutions of the MSC at p2 were placed into 10 to 20 replicates in 96-well plates in the presence of MesenPure (STEMCELL Technologies) and 0.5% penicillin-streptomycin. After 14 days, colony-forming unit-fibroblast (CFU-F) were performed using MesenCult Expansion Kit (STEMCELL Technologies®), where the wells were stained with Giemsa (Merck) according to manufacturer's instruction. Colonies were then counted manually. The frequency of progenitor cells in the undiluted starting population was calculated manually.

2.10 Microarray analysis

RNA of four HD- and four CML-derived MSCs was isolated using TRIzol™ Reagent (Thermo Fisher®) as previously described. RNA was hybridized on Human Clariom D Assay gene chip (Affymetrix®). Further steps, such as sample labeling, hybridization, washing and scanning were performed according to manufacturer's protocols. Downstream analysis was performed in R (v4.2.2), using packages oligo (v1.62.2), Biobase (v2.58), affycoretools (v1,70), limma (v3.54.2), PCAtools (v2.10), progeny (v1.20), msigdbr (v7.5.1) and clusterProfiler (v4.6) for analysis; data.table (v1.14.8), tibble (v3.2.1) and dplyr (v1.1.1) for data wrangling; and ggplot2 (v3.4.1), ggrepel (v0.9.2), ComplexHeatmap (v2.14), patchwork (v1.1.2) and cowplot (v1.1.1) for visualization.

Robust Multichip Average preprocessing was performed. This included background subtraction, quantile normalization and summarization. The latter step was performed at the probeset level. Principal Component Analysis was performed to evaluate if each sample group was clustering together (Supplementary Figure S3B). Samples CML2 and HD4 were deemed unfit for this analysis. CML2 was treated with imatinib which could be a confounding factor. HD4 was clustering apart from the other HDs and therefore considered an outlier. Non-mapping probes, probes mapping to sexual chromosomes and probes with missing data were filtered out. A PCA was again performed after the filtering (Supplementary Figure S3C).

Differential expression analysis was performed with limma-trend with an exclusion filter for probes with a mean expression below 5 on the log2 scale, note that this still retains 439,961 probes. A linear model for the CML vs. HD comparison was fit and its statistics were estimated with empirical Bayes moderation. *p*-values were corrected for multiple testing with the Benjamini–Hochberg procedure.

PROGENy was performed on the same data fed to the differential expression analysis and setting the "top" parameter to 1,000 and "scale" to false. PROGENy results were then scaled in regard to the HD samples. Over-Representation Analysis (ORA) and Gene Set Expression Analysis (GSEA) were performed on the results from the differential expression analysis. For ORA, the background probes considered were the same as the ones present in the differential expression analysis. Only enrichment for under-expressed probes was performed as no probe passed the over-expression significance threshold during differential expression analysis. For ORA and GSEA, *p*-values were corrected for multiple testing with the Benjamini–Hochberg procedure. Chosen custom gene sets and MSigDB gensets were evaluated separately. For PROGENy and GSEA, probe information was summarized down by mean to the gene level.

2.11 Statistical analyses

1-way or 2-way analysis of variance (ANOVA) were applied for multiple comparison analysis, with Tukey's or Bonferroni's post-hoc-test. Unpaired *t*-test was used to compare the means of two groups. Fisher's exact test was used for Contingency Table. Statistical calculations were performed with GraphPad Prism (GraphPad Software, La Jolla, CA, United States). Data are presented as mean ± standard deviation (SD). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ were considered statistically significant.

3 Results

3.1 Clinical characteristics of CML patients

Based on the conflicting reports on the frequency of MF, we first assessed the proportion of patients with MF at diagnosis in our CML cohort (n = 57). Here, 38.6% (n = 22) had MF at the time of diagnosis, with 31.6% having MF stage 1 (n = 18) and 7.0% (n = 4) having stage 2 (Figure 1A). No grade 3 MF was observed. As MF has previously been suggested to be an adverse prognostic marker in CML (Buesche et al., 2003; Buesche et al., 2004; Buesche et al., 2007) we started to compare different clinical parameters among patients

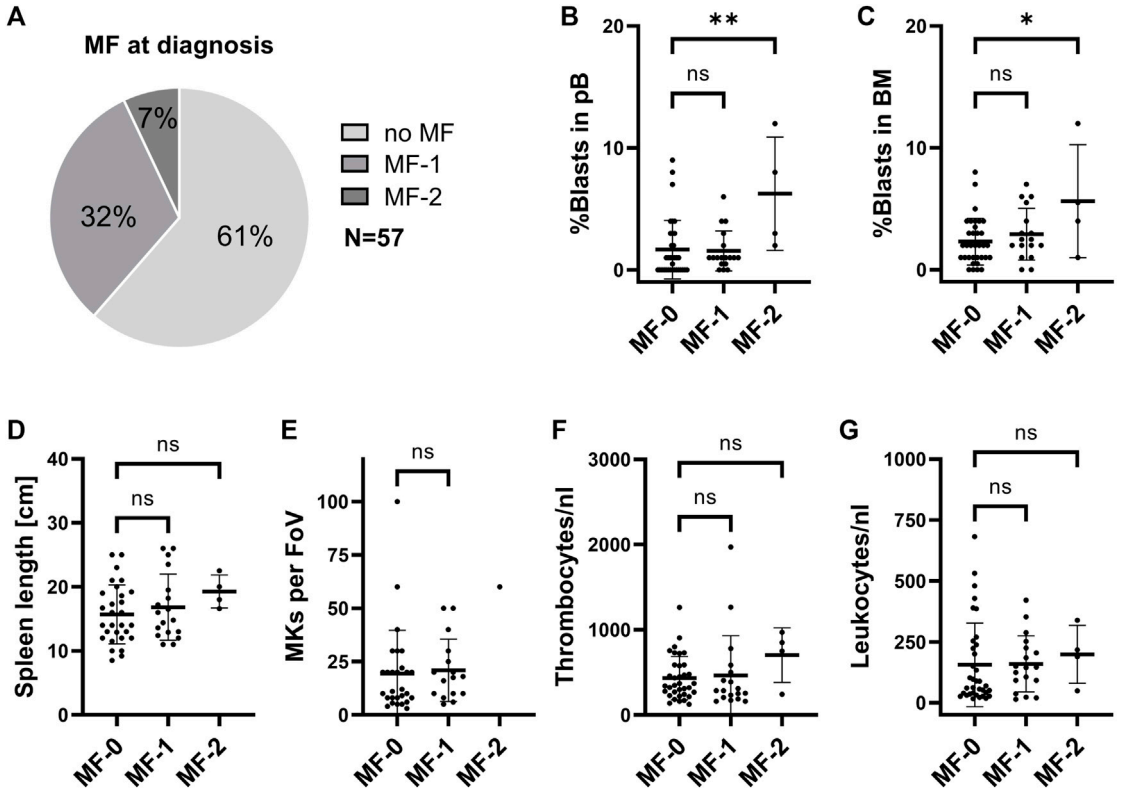

FIGURE 1
Clinical parameters in CML patients at initial diagnosis with different stages of BM fibrosis. **(A)** Proportion of CML patients with myelofibrosis (MF) in our cohort. **(B)** Peripheral blood (pB) blast cell count, **(C)** bone marrow (BM) blast cell count, **(D)** spleen length, **(E)** BM megakaryocytes (MKs), **(F)** pB thrombocytes and **(G)** pB leukocytes were assessed by the time of initial diagnoses and are represented, depending on the stage of MF. FoV: field of view.

with different stages of MF. Here we observed that the percentage of peripheral blood (pB) and BM blasts is significantly higher in CML patients with MF grade 2 (Figures 1B,C). Spleen length, the number of BM megakaryocytes (MKs), platelet counts and leukocyte counts did not significantly change upon MF development (Figures 1D–G). In our cohort, presence of MF also does not correlate with response to treatment at 12 months (Supplementary Figure S1A), risk scores (Supplementary Figure S1B–D) or patient age at diagnosis (Supplementary Figure S1E). Furthermore, TFR outcome also did not correlate with common risk scores (Supplementary Figure S1F–H).

3.2 Increase in megakaryocytes in α-SMA positive murine CML bone marrow upon TKI treatment

Using the transgenic CML model we previously confirmed that LSCs persist despite BCR::ABL1 inhibition (Schemionek et al., 2010; Hamilton et al., 2012). Further studies have revealed that CML development induces expansion of osteoblastic lineage cells and MF has been suggested to occur in these mice as analyzed by collagen (Schepers et al., 2013) or reticulin-staining (Reynaud et al., 2011). As the grade of fibrosis is usually low in CML, we here used α-SMA staining in order to dissect early fibrotic changes. To exclude any possibility of transgene expression in the non-hematopoietic compartment we first transplanted 2 × 10^6 BM cells, isolated from either *SCLtTA/BCR::ABL1* CML or *SCLtTA* control mice into 10 Gy irradiated recipients and allowed these cells to engraft for 1 week before inducing *BCR::ABL1* expression. 2 weeks after induction of the oncogene, mice were treated with either nilotinib (NIL) or vehicle (V) control for 3 weeks (Figure 2A). Upon autopsy the increase in spleen weight was evident in the CML V group compared to the control V group. This was largely reverted upon NIL treatment as expected and previously shown (Figure 2B). In line with these data, expression of *BCR::ABL1* was evident in V-treated CML mice but significantly decreased upon NIL treatment (Figure 2C). A detailed FACS immunophenotyping revealed that the B-cells were driving the disease phenotype and responded to TKI treatment (Supplementary Figure S2), as we had previously observed (Butow et al., 2023). Sternum sections of the BM were stained for α-SMA that is known to be expressed in myofibroblasts. Indeed, we observed a significant increase in V-treated CML mice that was reverted to some extend upon NIL treatment (Figures 2D,E). To our surprise, α-SMA appeared to be also expressed in CML MKs, which are a supposed driver of fibrotic transformation in MPN. FACS analysis revealed that MK percentages were not altered in the BM but increased in the spleen of CML transgenic mice and interestingly elevated in both organs upon TKI treatment (Figures 2F,G).

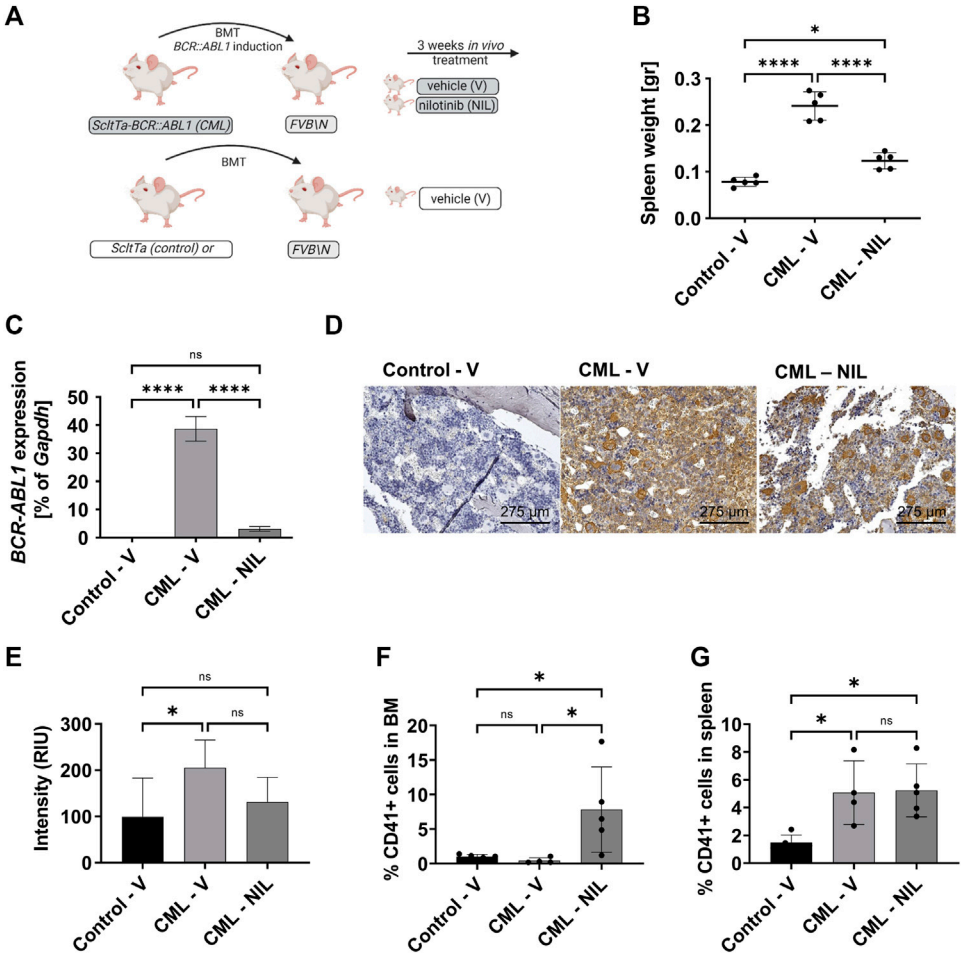

FIGURE 2
CML transgenic mice show increased megakaryocytes upon TKI treatment. **(A)** Schematic representation of the mouse model and experimental set up. **(B)** Spleen weight at endpoint analysis. **(C)** *BCR::ABL1* mRNA expression. Gapdh was used as housekeeping gene. **(D)** α-SMA staining of the sternum showing a representative example of each group. **(E)** Quantification of α-SMA staining. **(F)** FACS-immunophenotype of $CD41^+$ megakaryocytes in the bone marrow. **(G)** FACS-immunophenotype of $CD41^+$ megakaryocytes in the spleen. V: Vehicle; NIL: nilotinib; RIU: relative intensity unit. $*p < 0.05$, $****p < 0.0001$.

3.3 Phenotypic profiling of CML MSCs

Mesenchymal stromal cells (MSCs) were either isolated from BM of CML patients or the hip bone of HDs via plastic adherence (Figure 3A) and their immunophenotype was analyzed at p2 (Figure 3B). The FACS analyses showed the expression of common MSC markers, such as CD73, CD90, CD14 and CD44. Typical hematopoietic markers, such as CD34, CD45, CD11b and HLA-DR were not present, consistent with the characteristic pattern of mesenchymal surface markers. Next, we evaluated their differentiation potential into the adipogenic, osteogenic, and chondrogenic lineage (Figure 3C). The adipogenic potential of CML-derived MSCs was significantly enhanced compared to HD. The osteogenic differentiation of CML-derived MSCs appeared to be elevated but that was rather due to a single CML sample showing an outstanding osteogenic capacity. Chondrogenic differentiation did not differ in CML vs. HD-derived samples. In order to evaluate the clonogenic capacity of CML MSCs, we performed a limiting dilution CFU-F assays (Figure 3D). These data show that there was no difference observed in CML-vs HD-derived MSCs. Overall, although CML-derived MSCs showed an increased capacity to differentiate into the adipogenic lineage, no other substantial phenotypic differences were observed in the two groups.

3.4 CML MSCs are driven towards a fibrotic and potentially LSC-supporting expression profile

To determine the gene expression signature of CML-vs HD-MSCs, we performed a microarray analysis. Overall changes where rather mild in these MSCs that were expanded until p2, as shown by the volcano plot (Supplementary Figure S3A). However, initial quality control showed specific clustering of CML vs. HD samples and revealed HD 4 as an outlier (Supplementary Figure S3B), potentially due to technical reasons and was therefore excluded from subsequent analysis. In addition, CML 2 was previously treated and therefore likewise excluded from further analyses (Supplementary Figure S3C). We could observe significant

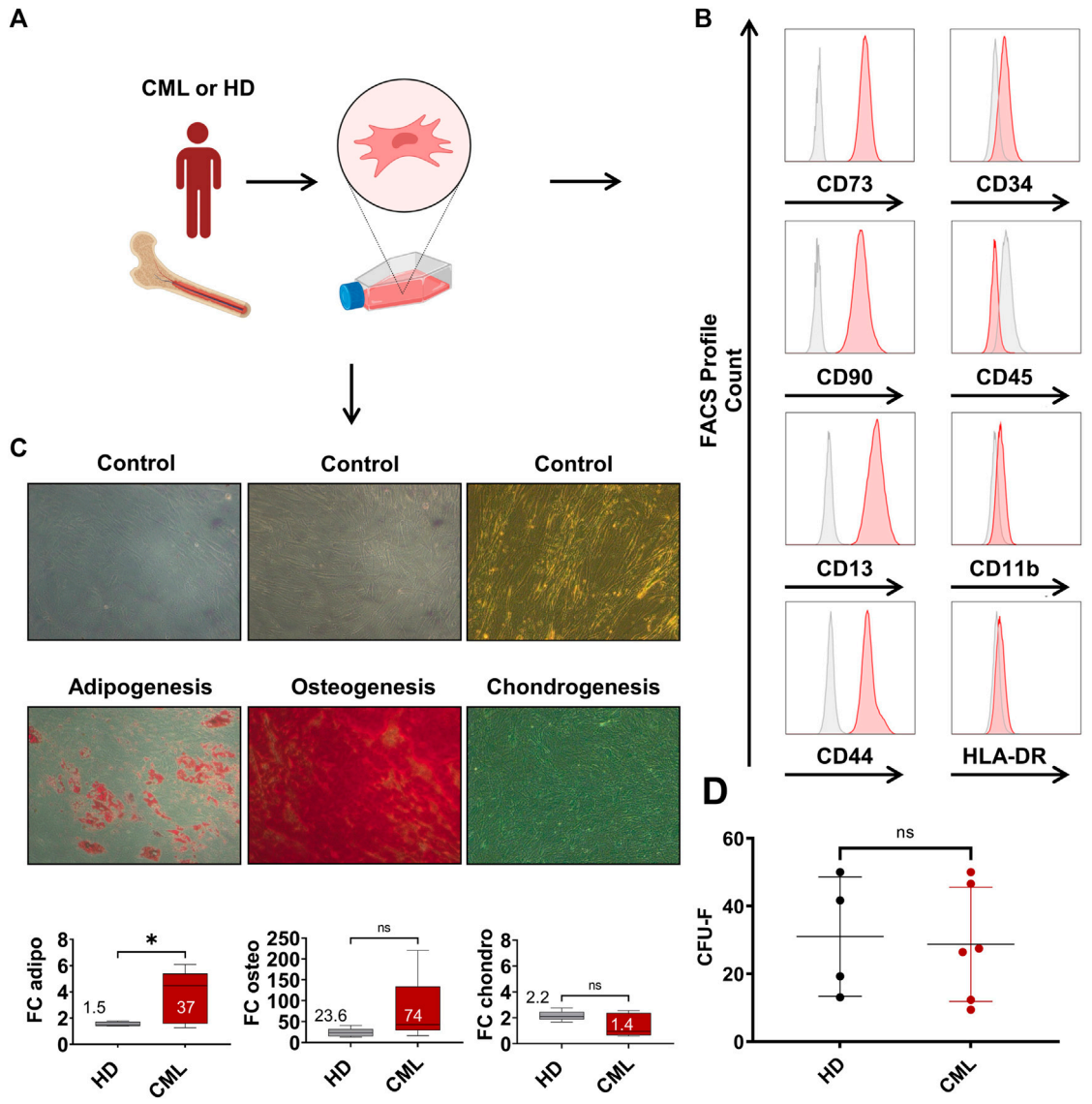

FIGURE 3
Characterization of CML MSCs shows increased differentiation, but unaltered clonogenic potential. **(A)** MSCs were extracted from the BM and subsequently isolated via plastic adherence. **(B)** MSC FACS profile of MSCs in p2. **(C)** Healthy donor- (HD) CML-derived MSCs were analyzed for their potential to differentiate towards the adipogenic, osteogenic, or chondrogenic lineage (n = 5/5). Fold change (FC) over control of the absorbance is reported in the box plot at the bottom of panel **(C)**. Mean FC is indicated in the graph. **(D)** CFU-F of HD-vs CML-derived MSC. *$p < 0.05$.

changes in a defined fibrotic gene signature set, which was previously derived from the analysis of expression profiling datasets of human organs with fibrosis-related diseases (Wang et al., 2020) (Figure 4A). Furthermore, an altered expression profile was also observed in a defined gene set for hematopoiesis support (Figure 4B). Next, gene set expression analysis (GSEA) was performed using the molecular signature database (MSigDB) hallmark gene sets collection (Figure 4C). A significant alteration of signaling pathways involved in inflammation was found, such as interferon gamma and alpha response. Interestingly, a significant upregulation of epithelial-to-mesenchymal transition (EMT) signature was also observed. Among the downregulated pathways (Figure 4D), another gene set of high interest that was significantly downregulated was the one containing genes with at least one occurrence of the motif detected by MIR431 (Jiang et al., 2018). This microRNA has been previously found downregulated in AML and its overexpression induced downregulation of TGF-β as well as epithelial-to-mesenchymal transition-related genes. Finally, we show 14 additional signatures of pathway perturbation (pathway responsive genes, PROGENy analysis) (Schubert et al., 2018) in our two cohorts and found a significant upregulation of the TGF-β pathway in the CML-derived MSCs (Figure 4E).

3.5 TFR often fails in patients with MF at diagnosis

The success of TFR is compromised by the persistence of LSCs in CML patients on TKI therapy, which re-expand upon

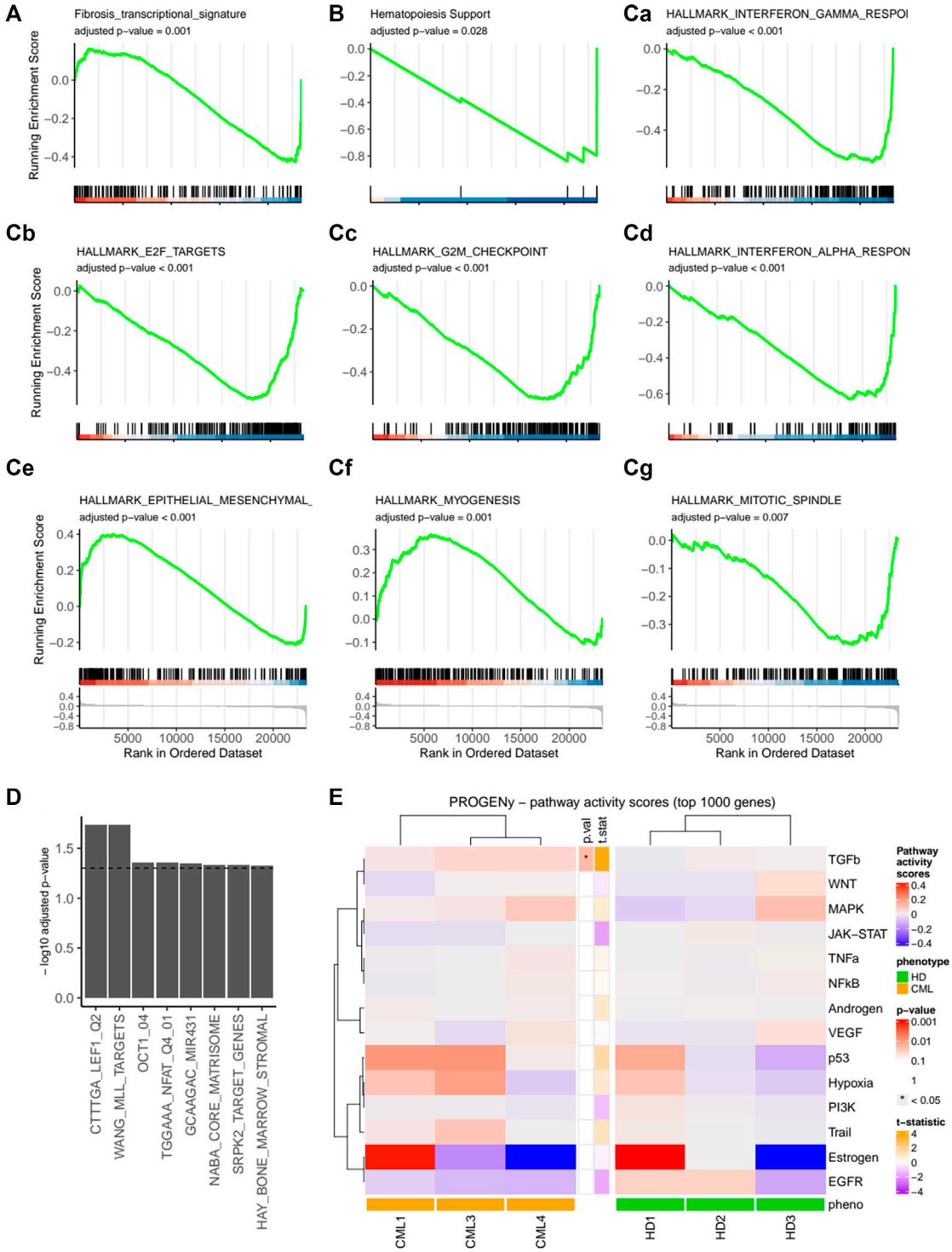

FIGURE 4
Expression profile of the mesenchymal CML fibrotic niche. HD-MSCs or CML-MSCs were expanded until passage 2 and RNA expression analysis was performed using a microarray. GSEA of custom gene sets for fibrosis **(A)** and hematopoiesis support **(B)** in CML vs. HD are shown. In **(C)**, hallmark gene sets with adjusted *p*-value < 0.05 are shown. Specifically, Ca shows Interferon gamma response, Cb E2F targets, Cc G2M checkpoint, Cd Interferon alpha response, Ce epithelial-to-mesenchymal transition, Cf myogenesis, Cg mitotic spindle. **(D)** Selection of significant (adjusted *p*-value < 0.05) Molecular Signatures Database (MsigDB) gene sets for over-representation analysis of downregulated genes in CML vs. HD. **p* < 0.05. **(E)** Pathway responsive genes (PROGENy) of multiple comparisons of HD-MSCs or CML-MSCs. Upregulated pathways are shown in red and downregulated ones in blue.

TABLE 1 Characteristics of patients that discontinued TKI.

No.	Age	Sex	MF grade	TFR success	TKI	Years of TKI	Years of MR4-MR5	Years of TFR	Study
1	57	male	0	Yes	Nilotinib	3	2.75	8.5	EnestFreedom
2	39	male	0	Yes	Nilotinib	5	>2	6.5	EnestFreedom
3	48	male	0	Yes	Imatinib	12	9	2	-
4	64	female	0	Yes	Nilotinib	8	3.5	1.25	-
5	58	female	0	Yes	Bosutinib	5	3.5	4	Endure
6	21*	female	0	Yes	Nilotinib	(3 &) 6	2.5	5	(EnestFreedom) Endure
7	58*	male	1	No	(Imatinib) Nilotinib	(3 &) 8	4	-	(EuroSki) NAUT
8	59	male	1	Yes	Nilotinib	3	2	6.25	CML-V/TIGER
9	57	female	1	No	Imatinib	8	5	-	-
10	57	female	1	Yes	Nilotinib	4.5	3.5	6	-
11	60**	male	1	No	Bosutinib	2.5	16 months MR3	-	-
12	74	male	2	No	Imatinib	4	3	-	-
13	60	female	2	No	Nilotinib	4	1.5	-	CML-V/TIGER

*Patients attempted discontinuation of tyrosine kinase inhibitor (TKI) two times at different timepoints as indicated and did not achieve therapy free remission (TFR) the first time.
**Patient only reached MR3 before TKI discontinuation and was not included in final analysis.
No.: number, MF: myelofibrosis. Age, sex and MF grade are given for the timepoint of diagnosis, the other parameters for the timepoint of TKI discontinuation. TFR success was evaluated as described in Material and Methods.

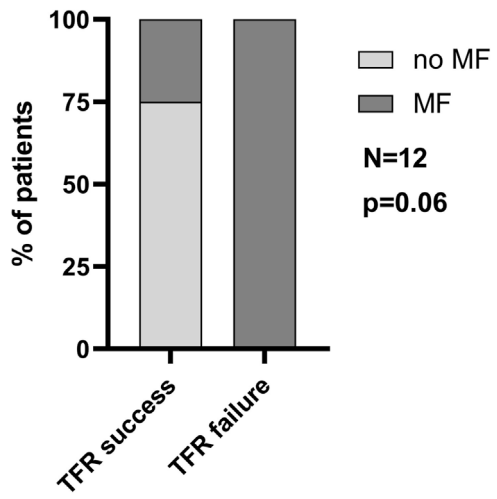

FIGURE 5
Presence of myelofibrosis at initial diagnosis is associated with TFR failure. The percentage of patients with or without MF that successfully reached TFR. N = 12.

TKI discontinuation. Based on the expression analysis of primary patient-derived MSCs indicating LSC-supporting potential, we investigated the impact of MF on TFR. A minority of CML patients underwent TKI discontinuation. In our cohort of patients with available MF grading at diagnosis, 12 patients discontinued TKI therapy after at least 1.5 years of MR4 or better. The clinical parameters of these patients are described in Table 1 and Supplementary Table S1. In the small cohort of patients, no significant differences in age, treatment duration or duration of MR4/5 were observed when comparing patients who succeeded or failed TFR. Of those 12 patients, 4 failed TFR (33%) and interestingly, all presented with MF at diagnosis (Figure 5). There was one additional patient with MF grade 1 at diagnosis who failed TFR, but he was excluded from this analysis, as he only reached MR3 for 16 months and therefore did not fulfil the current criteria for TKI discontinuation. Out of 8 patients who achieved TFR, 2 (25%) had MF grade 1 at diagnosis, whereas the other 6 (75%) had no BM fibrosis. Notably, all patients without MF at diagnosis achieved TFR, while all patients with MF grade 2 at diagnosis, failed. Only patients with MF grade 1 varied in TFR success, half of them failing, the other succeeding (Table 1).

4 Discussion

In this study, we suggest a potential link between the presence of BM fibrosis in CML patients at diagnosis and persistence of LSCs that are capable to re-expand upon TKI discontinuation, thereby resulting clinically in TFR failure.

First, we could observe that in our cohort around 40% of the patients presented with MF at diagnosis and MF grade 2 correlated with higher blast counts in the blood and BM. Although the impact of BM fibrosis on prognosis was considered adverse in the pre-TKI era (Lazzarino et al., 1986; Kvasnicka et al., 2001; Buesche et al.,

2003), it seems to be less relevant nowadays, due to the enormous success of TKIs and the drastic improvements in patient management that were introduced (Kantarjian et al., 2005; Tanrikulu Simsek et al., 2016). BM fibrosis at diagnosis has been shown to revert in the majority of patients upon TKI treatment (Buesche et al., 2007; Eliacik et al., 2015; Narang et al., 2017) and we likewise observed this in patient 11. Tanrikulu Simsek et al. (2016) furthermore confirmed a decrease in fibrosis grades after long-time imatinib treatment. It has to be noted that in a few single patients imatinib treatment was associated with induction of BM fibrosis (Buesche et al., 2007; Shanmuganathan et al., 2019) and interestingly, in patient 3 of our cohort we see development of low-grade fibrosis after 5.5 years of imatinib treatment. A potential mechanism of the antifibrotic properties of TKIs involves targeting of the platelet-derived growth factor receptor protein kinase α and β (Hantschel et al., 2008), which are both involved in the process of fibrogenesis. Moreover, TKIs bind and inhibit ABL itself, which is part of the signaling cascade of TGF-β (Daniels et al., 2004), another important mediator of fibrosis development. However, to our knowledge, the origin of fibrosis in CML has not been addressed so far.

In Ph^- MPNs, MKs and MSCs are known to play key roles in the development of fibrosis (Malara et al., 2014; Decker et al., 2017; Schneider et al., 2017; Kramann and Schneider, 2018). In the BM of our SCLtTA-*BCR::ABL1* mice, high levels of α-SMA were detected, which is an early marker of fibrogenic activity (Bochaton-Piallat et al., 2016). This expression was partly reverted by nilotinib treatment *in vivo*, mirroring the findings of others that TKI treatment in CML can revert MF (Eliacik et al., 2015). Surprisingly, α-SMA was also expressed by MKs in treated and untreated CML mice, and their numbers were increased in the spleen of CML transgenic mice and elevated in both BM and spleen upon nilotinib treatment. Interestingly, it has been suggested that embryonic $CD41^+$ cells from the aorta-gonad-mesonephros expressed mesenchymal markers and displayed myofibroblastic potential and could differentiate into α-SMA-positive cells (Zhou et al., 2012). MKs are found increased in CML (Theologides, 1972; Thielen et al., 2011; Mondet et al., 2015; Hussein et al., 2017) and their numbers positively correlate with the grade of fibrosis in the BM of CML patients (Thiele et al., 2000; Rao et al., 2005).

The impact of this increase of MKs requires further study, not only to address their potential role as a driver of MF in CML, but also to assess whether they might directly support LSC persistence. It has been shown that malignant MKs produce TGF-β, which supports leukemogenic capacity of CML cells (Tanabe et al., 2020), and also, MK-derived expression of TGF-β and CXCL4 is known to maintain HSC quiescence (Bruns et al., 2014; Zhao et al., 2014). Moreover, we and others have previously shown that potent LSCs also reside in the CML spleen (Schemionek et al., 2012; Buhrer et al., 2022) and combined with the splenic increase in MKs, this further supports the rationale to study MK-driven LSC persistence. In line with the observation of Tanabe et al. (2020), where in CML BM-MKs correlated with *BCR::ABL1* copy number after TKI treatment, we could speculate that the persistence of this MK population might represent an obstacle for the success of TFR.

With regard to the role of MSCs in fibrosis development, we observed that MSCs from CML patients increasingly differentiate into adipogenic and possibly osteogenic cell types. MSCs with an adipogenic and osteogenic gene expression pattern have been shown to promote fibrosis in mice (Schepers et al., 2013; Leimkuhler et al., 2021) and a beneficial role of the normal adipo-/osteogenic niche in the maintenance and regulation of normal SC (Omatsu et al., 2010) and LSCs (Schepers et al., 2013) has been shown. The increased adipogenic potential observed in our CML-derived MSCs might suggest a role of adipocytes in the maintenance of LSCs, although the role of marrow adipose tissue in malignant hematopoiesis is still not completely understood. A possible hypothesis is that adipocytes might sustain the survival and proliferation of blasts by supplying them with free fatty acid, similarly to what has been described in AML (Shafat et al., 2017). Moreover, under steady state and metabolic stress conditions, BM adipocytes have been identified as major supplier of stem cell factor, which both malignant and healthy HSC critically depend on (Agarwal et al., 1995; Zhou et al., 2017; Zhang et al., 2019). Furthermore, pre-adipocytic MSCs have been shown to highly express stem cell supporting factors (Matsushita et al., 2020). On the other hand, the presence of mature osteoblasts has been shown to impair Ph^- MPN development (Krevvata et al., 2014) revealing that the complexity of the underlying mechanism is hardly understood. MSCs have been previously shown to support LSCs in CML. E.g., it was shown that C-X-C motif chemokine ligand 12 (CXCL12) deletion from mesenchymal progenitors enhanced sensitivity of LSCs to TKI treatment and downregulated mechanisms associated with LSC quiescence (Agarwal et al., 2019). Moreover, BM MSC gene profiling in CML patients at diagnosis and in DMR showed that LSC supporting genes were upregulated despite TKI treatment (Aggoune et al., 2017), further reinforcing that MSCs play a fundamental role in supporting LSC persistence.

When we studied the gene expression profile of CML-MSCs, we found that the signature reflecting fibrosis and hematopoiesis support was altered. In particular, the activation of TGF-β signaling in MSCs might be of interest, since TGF-β is both involved in fibrosis development and in supporting LSC persistence in CML, further tying fibrosis and LSC persistence together (Naka et al., 2010). Furthermore, expression of the gene set MIR431 was downregulated in our analyses. MIR431 has previously been shown to be downregulated in AML and overexpression induced downregulation of TGF-β and EMT markers like fibronectin, α-SMA, and vimentin (Jiang et al., 2018). EMT was likewise significantly regulated as one of the hallmark gene sets tempting to speculate that EndMT (endothelial-to-mesenchymal transition) could potentially be a further source of mesenchymal cells, as previously been suggested in Ph^- MPN (Erba et al., 2017).

It is important to mention that the association between persistence of LSC and failure of TFR is still matter of debate. The persistence of LSC in the BM is likely guaranteed by a combination of factors, such as LSC-intrinsic survival pathways, protection provided by the BM niche as well as immune control [Reviewed in (Soverini et al., 2021)]. Importantly, LSC are known to hijack the BM niche, which in turn will nurture malignant cells at the expense of normal HSC. For example, altered levels of pro-inflammatory cytokines or chemokines that favor LSC self-renewal, are found in CML BM even upon TKI-induced

remission (Zhang et al., 2012; Zhang et al., 2016). If changes occur in the processes governing the balance between LSC and healthy HSC, LSC might find themselves in a fostering soil for their expansion.

A final link between MF and LSC persistence became evident when we further analyzed our cohort of CML patients who reached the requirements for TKI discontinuation. Specifically, we could observe an association between BM fibrosis and TFR outcome, since the totality of patients that failed TFR had MF at diagnosis.

Nevertheless, it is important to underline that this correlation was not statistically significant ($p = 0.06$) and further studies on larger cohorts are necessary to confirm this association.

Moreover, another important aspect to discuss is that according to the literature, the correlation between the common risk scores and TRF success is not always evident. In some studies, patients with low-to intermediate-risk Sokal score had a significantly higher probability of persisting complete molecular response compared to those with high-risk score (Mahon et al., 2010; Yhim et al., 2012). In our study and in Ross *et al* (Ross et al., 2013), no significant correlation was observed.

In conclusion, in our hypothesis the BM fibrosis in CML patients likely recedes upon TKI treatment, to a macroscopically undetectable level. Nevertheless, such a process fundamentally impacts on the BM niche and one could hypothesize that specific changes might remain on a molecular level, which could drive LSC re-expansion. However, this needs further comparative studies, ideally at the single cell level, of the LSC niche in patients who either relapse after discontinuation of the TKI or who do not.

However, as the grading of MF seems to vary significantly depending on the individual assessment, another, possibly technology-aided and centralized approach to grade fibrosis in the BM would help to reduce the variations seen in different studies (Gralnick et al., 1971; Kvasnicka et al., 2001; Buesche et al., 2003; Hamid et al., 2019). One major limitation of our study is that in our cohort, only a small number of patients in TFR could be included. Therefore, larger studies involving centralized MF evaluation are certainly needed for more robust conclusion on the impact of MF on TFR outcome. However, our data suggest that this might bear the potential to significantly improve the prediction of TFR outcome and moreover, suggest follow-up studies on the underlying mechanisms of fibrotic transformation in CML in the context of LSC persistence.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE230534.

Ethics statement

The studies involving human participants were reviewed and approved by local ethics board of the medical faculty RWTH Aachen (EK 206/09 and EK 391/20). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Local Authorities Of North Rhine-Westphalia, Germany (LANUV Az. 84-02.04.2013.A072).

Author contributions

HJ and MV designed, conducted and discussed experiments, analyzed data, designed figures and wrote the manuscript. MB, CN, and LF designed, conducted and discussed experiments. IC and TM supported the bioinformatic analysis of the microarray. NC, MH, TB, and WW discussed experiments and analyzed data. FB and MC provided clinical samples and expertise. MS designed and discussed experiments, analyzed data, designed figures and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

TB served as a consultant or speaker at satellite symposia for AstraZeneca, Janssen, Merck, Novartis and Pfizer and received research funding form Novartis and Pfizer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1212392/full#supplementary-material

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Multidisciplinary management in chronic myeloid leukemia improves cardiovascular risk measured by SCORE

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Introduction: Cardiovascular events are one of the main long-term complications in patients with chronic myeloid leukemia (CML) receiving treatment with tyrosine kinase inhibitors (TKIs). The proper choice of TKI and the adequate management of risk factors may reduce cardiovascular comorbidity in this population.

Methods: This study evaluated the cardiovascular risk of a cohort of patients with CML at diagnosis and after follow-up in a specialized cardiovascular risk consultation. In order to do this, we performed data analysis from 35 patients who received TKIs and were referred to the aforementioned consultation between 2015 and 2018 at our center. Cardiovascular risk factors were analyzed separately, as well as integrated into the cardiovascular SCORE, both at diagnosis and at the last visit to the specialized consultation.

Results: At the time of diagnosis, 60% had some type of risk factor, 20% had a high or very high risk SCORE, 40% had an intermediate risk, and 40% belonged to the low risk category. During follow-up, the main cardiovascular adverse event observed was hypertension (diagnosed in 8 patients, 23%). 66% of patients quit smoking, achieving control of blood pressure in 95%, diabetes in 50%, weight in 76%, and dyslipidemia in 92%. 5.7% of patients suffered a thrombotic event and a significant percentage of patients showed a reduction in their SCORE.

Conclusion: Our study shows the benefit of controlling cardiovascular risk factors through follow-up in a specialized consultation for patients with CML treated with TKI.

KEYWORDS

chronic myeloid leukemia (CML), cardiovascular risk, tyrosine kinase inhibitor (TKI), SCORE, multidisciplinary management, coronary disease, thromboembolic disease

1 Introduction

The introduction of tyrosine kinase inhibitors (TKIs) in the treatment of chronic myeloid leukemia (CML) marked a significant change in the management and prognosis of this disease (Berman, 2022). This family of drugs allowed higher survival rates of these patients to a level like that of the general population (Hochhaus et al., 2020). Moreover, TKIs

helped in achieving symptom control, total clearance of the tumor clone, and significantly reducing the rate of acute transformation (Cortes et al., 2021).

However, TKI treatment poses new challenges in the management of CML, like those associated with the numerous interactions of these drugs and the adverse effects derived from their use (Haouala et al., 2011). Among the latter, the most frequent and concerning are cardiovascular side effects (Douxfils et al., 2016) (Dahlén et al., 2016), which raise the need for strict control of cardiovascular risk factors at the time of diagnosis or those emerging over the follow-up (Barber et al., 2017).

Currently, five TKIs with similar efficacy rates and a different toxicity profile are approved for the treatment of CML (García-Gutiérrez & Hernández-Boluda, 2019). Generally, patients experience some type of (mostly mild) adverse effect, that may sometimes prompt a change in TKI (Cortes & Kantarjian, 2016).

The mechanism by which TKIs cause cardiovascular damage is not fully characterized, although it appears to be related to endothelial damage through non-specific inhibition of tyrosine kinases ("off-target" effect), alteration of glycemic metabolism, direct hypertensive effect or glomerular impairment (Chaar et al., 2018).

There are no studies comparing directly second-generation TKIs (dasatinib, nilotinib, bosutinib), but the results of studies comparing these with imatinib show a higher rate of cardiovascular events with this generation of TKIs, so imatinib may be a preferable option in patients with a high risk of cardiovascular disease (Cortes, 2020).

Furthermore, no clear consensus exists on when to refer a patient with CML from the hematology consultation to another specialist for the evaluation and management of cardiovascular risk. Guidelines on this matter recommend doing so in the case of a history of cardiovascular disease (Seguro et al., 2021), high risk of cardiovascular disease (Zamorano et al., 2016) or presence of risk factors when starting high risk TKI such as nilotinib (NCCN Clinical Practice Guidelines in Oncology, 2023). There are no specific recommendations to this effect from the European Leukemia Net.

However, at the time of diagnosis, patients diagnosed with CML presented a high prevalence of cardiovascular risk factors, which seems to be higher than that of the general population (Seguro et al., 2021). One study showed, at the time of CML diagnosis, a prevalence of 30% of hypertension, 11% of diabetes and 18% of dyslipemia (Coutinho et al., 2017).

Most of our knowledge about the efficacy and adverse effects of TKIs comes from clinical trials. Nevertheless, their results could underestimate the development of cardiovascular comorbidity, considering the exclusion of patients with insufficient control of cardiovascular risk factors, or the younger average age of patients included in the main first-line trials with dasatinib (Kantarjian et al., 2010) or nilotinib (Saglio et al., 2010).

Therefore, real world evidence studies are essential, as they are able to show the prevalence of complications arising from the use of TKIs in a routine clinical practice scenario. One of the largest studies to date (Coutinho et al., 2017), showed a prevalence of almost 80% of cardiovascular risk factors at 5 years after the diagnosis of CML.

In this study, we report our experience in the management of cardiovascular risk factors at our center, where patients are referred to a specialized internal medicine consultation at diagnosis or during follow-up. The purpose of this strategy is to optimize the control of cardiovascular risk factors. Only symptomatic patients are referred to other specialized consultation (cardiology or angiology).

To analyze the impact of this intervention, we have used the SCORE (Systematic Coronary Risk Evaluation) model, which estimates the risk of death from cardiovascular causes in 10 years. It has the advantage of being adjusted to different European countries, and estimates mortality associated with all atherothrombotic manifestations and not just coronary mortality, unlike the Framingham score. Moreover, SCORE is straightforward to calculate because it includes few parameters: age, sex, systolic blood pressure, total cholesterol and smoking (Visseren et al., 2021).

This model has already been used by other researchers to evaluate the risk of developing cardiovascular events in patients with CML treated with different TKIs, demonstrating its predictive value at diagnosis (Breccia et al., 2015; Caocci et al., 2019).

2 Materials and methods

2.1 Study design

This is a retrospective, single-center observational study that analyzed a total of 35 patients diagnosed with CML at the 12 de Octubre University Hospital, referred to the cardiovascular disease consultation between 2015 and 2018, who received treatment with one of the approved TKIs for this indication (imatinib, dasatinib, nilotinib, bosutinib and ponatinib). The patients received outpatient follow-up in hematology consultation and by an internal medicine specialist in the aforementioned cardiovascular control consultation.

The diagnosis of CML was made following criteria established by the latest classification of hematological neoplasms published by the WHO (Swerdlow et al., 2017). The following prognostic scores for CML were applied to the diagnosis: Sokal, Hasford, EUTOS and ELTS. Regarding the criteria used to define cardiovascular risk factors, they are explained below.

2.2 Cardiovascular variables

Arterial hypertension: defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, following the criteria used by the ESC/ESH Guidelines for the management of arterial hypertension (Williams et al., 2018). Arterial hypertension was considered to be controlled according to the target for general and specific subgroups of hypertensive patients, following the mentioned guidelines.

Dyslipidemia: defined as hypertriglyceridemia (triglycerides level >200 mg/dL) and/or hypercholesterolemia (cholesterol level >200 mg/dL), following the criteria from the ESC/EAS Guidelines for the management of dyslipidaemias (Mach et al., 2020). Dyslipidemia was considered to be controlled following the criteria defined by these guidelines.

Diabetes mellitus: defined as an A1C ≥ 6.5%; fasting blood glucose ≥126 mg/dL; blood glucose ≥200 mg/dL 2 hours after a 75 mg intake of glucose; or a casual blood glucose ≥200 mg/dL, according to the ESC Guidelines on diabetes (Cosentino et al., 2020).

TABLE 1 Baseline characteristics of patients (n = 35).

Gender	
Male, n (%)	19 (54.3%)
Female, n (%)	16 (45.7%)
Median age at diagnosis (SD)	50.51 years (13.57)
Sokal Index	
Low, n (%)	18 (51.4%)
Intermediate, n (%)	9 (25.7%)
High, n (%)	8 (22.9%)
Hasford Score	
Low, n (%)	21 (60%)
Intermediate, n (%)	10 (28.6%)
High, n (%)	2 (5.7%)
Unknown, n (%)	2 (5.7%)
EUTOS Score	
Low, n (%)	6 (17.1%)
High, n (%)	27 (77%)
Unknown, n (%)	2 (5.7%)
ELTS Score	
Low, n (%)	21 (60%)
High, n (%)	12 (34.3%)
Unknown, n (%)	2 (5.7%)

TABLE 2 TKI (tyrosine kinase inhibitor) received for CML.

Imatinib, n (%)	33 (94.3%)
Suspension	20 (60.6%)
Reasons	
Lack of response	8 (40%)
Toxicity	7 (35%)
Clinical Trial	5 (25%)
Dasatinib, n (%)	16 (45.7%)
Suspension	8 (50%)
Reason	AE: 8 (100%)
Nilotinib, n (%)	15 (42.86%)
Suspension	9 (60%)
Reasons	
Lack of response	1 (11.11%)
Toxicity	8 (88.89%)
Bosutinib, n (%)	3 (8.6%)
Suspension	1 (33.34%)
Reason: Toxicity	1 (100%)
Ponatinib, n (%)	1 (2.9%)
Suspension	1 (100%)
Reason: Clinical Trial	1 (100%)
TKI, total number used during follow-up, n (%)	
1	15 (42.8%)
2	10 (28.6%)
3	9 (25.7%)
4	1 (2.9%)

Control of diabetes was defined according to the targets specified by these guidelines.

Alcohol abuse: defined following criteria from DSM-V (APA, 2013).

SCORE (Systematic Coronary Risk Evaluation): defined following ESC criteria (Visseren et al., 2021).

2.3 Statistics

Frequencies were calculated as percentages for qualitative variables and as means and standard deviations for quantitative variables. Comparison of variables was carried out using the McNemar-Broker test. A $p < 0.05$ was considered statistically significant. Statistical analysis was conducted using the SPSS computer program version 25.0 (IBM, Chicago, IL).

3 Results

Table 1 summarizes the main characteristics of the 35 patients included in the study. The mean age at the time of referral to the cardiovascular control consultation was 50 years (standard deviation, 13.5). 45.7% of patients were women. Over half of the patients (51.4%) were classified in the low risk category according to the Sokal index, 60% according to the Hasford score, and 60% according to the ELTS score. However, most patients belonged to the high risk group according to the EUTOS Score (77.1%).

Regarding the prescribed TKI (Table 2), all except 2 patients received imatinib (median time of exposition, 20.3 months), 45.7% received dasatinib (median time of exposition, 24 months), 42.9% received nilotinib (median time of exposition, 15.5 months), 3 patients received bosutinib (median time of exposition 4.1 months), and 1 received ponatinib (7.3 months of exposition). 60.6% of patients treated with imatinib had to stop it (in 40% of these cases due to lack of optimal response, 35% as a result of adverse effects, and 25% because of clinical trial protocol). Patients who stopped dasatinib (57.7% of those who received this drug) did so for reasons related to adverse effects. The discontinuation rate with nilotinib was 60% (in one because of lack of efficacy and in the remaining 88.9% as a consequence of toxicity). Out of the three patients treated with bosutinib, one stopped it due to toxicity, and the only patient treated with ponatinib stopped it because of clinical trial protocol.

TABLE 3 Cardiovascular Risk Factors at first visit and last follow-up in Specialized Consultation.

Months from diagnosis to referral, mean (SD)	53.80 (75.1)	
Smoking, n (%)	At first visit	At last follow-up
Active	6 (17.14%)	2 (5.71%)
Previous	10 (28.57%)	14 (40.00%)
No smoking	19 (54.29%)	19 (54.29%)
Alcohol abuse, n (%)	4 (11.4%)	2 (5.7%)
Arterial Hypertension, n (%)	12 (34.3%)	20 (57.14%)
Improvement in blood pressure levels		20 (100%)
Control of hypertension		19 (95%)
Diabetes mellitus type 2, n (%)	3 (8.6%)	6 (17.2%)
Improvement in glucose levels		6 (100%)
Control of diabetes		3 (50%)
Dyslipidemia, n (%)	14 (40%)	24 (68.57%)
Improvement in lipid levels		24 (100%)
Control of dyslipidemia		22 (91.66%)
Cardiovascular Disease, n (%)	7 (20.00%)	11 (31.4%)
Coronary Disease	2 (5.7%)	3 (8.57%)
Cerebrovascular Disease	2 (5.7%)	2 (5.7%)
Peripheral Arterial Disease	3 (8.6%)	6 (17.2%)
COPD	1 (2.90%)	1 (2.9%)
Chronic kidney disease	2 (5.70%)	2 (5.7%)

SD: standard deviation; COPD: chronic obstructive pulmonary disease

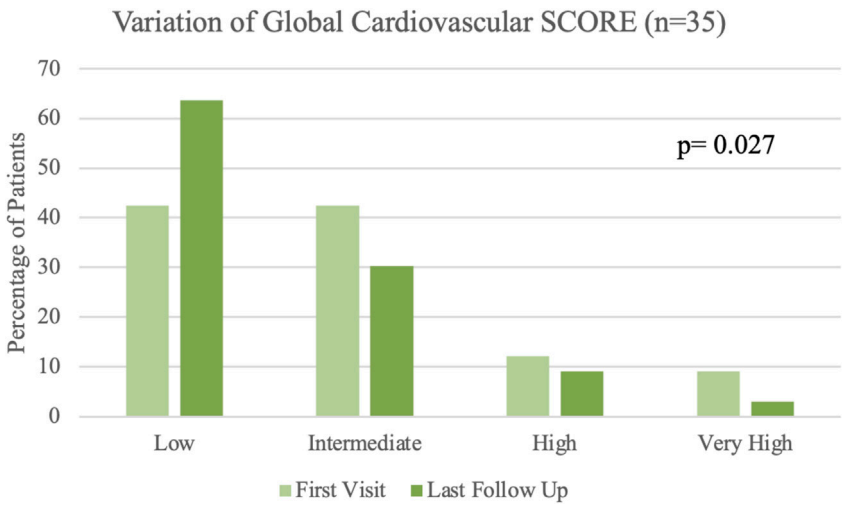

FIGURE 1
SCORE at the time of the first visit and at last follow up.

Table 3 shows the proportion of patients who had some type of cardiovascular risk factor either at the time of referral or at the last visit to the cardiovascular control consultation. The time elapsed between diagnosis and consultation was approximately 54 months on average. At the time of the consultation 17.1% had an active tobacco habit and 28.6% had stopped smoking. 11.4% had alcohol consumption in the range of abuse according to the previously stated criteria. 34.3% had hypertension, 8.6% had DM, and 40% had dyslipidemia. Seven patients had already developed cardiovascular disease at the time of the consultation (2 in the form of coronary disease, 2 stroke, and 3 peripheral arterial obstructive disease). One patient had a diagnosis of chronic obstructive pulmonary disease COPD and 2 had chronic kidney disease.

During a mean follow-up of 31.25 months, 3 patients were diagnosed with diabetes, 8 developed hypertension (13.3% of patients with nilotinib, 12.5% with dasatinib and 12.1% with imatinib), 10 dyslipidemia and 3 peripheral arterial obstructive disease (PAOD). Strict control of hypertension was achieved in all but one patient, control of dyslipidemia in all but 2 and only 3 patients did not reach adequate diabetes control. However, there was an improvement of blood pressure, glucose level and lipids in all patients. In 12 patients, it was necessary to change either the type or dosage of TKI because of interactions with concomitant medication, with statins being the main reason in 75% of these cases.

The most frequent cardiovascular disease in our cohort was PAOD (6 patients developed PAOD after CML diagnosis, 3 of them before referral to Internal Medicine Department and 3 of them afterwards). The median age at the time of PAOD diagnosis was 63.5 years, with a median time of 13.15 years from the introduction of TKI treatment. Regarding the former 3 patients, 2 were receiving nilotinib and 1 dasatinib. Two of them belonged to the intermediate risk and 1 to the very high risk SCORE category. An improvement of SCORE was reached in 2 of them.

The latter 3 cases with PAOD were diagnosed with a median of 2.5 years after the first consultation. All of them were receiving imatinib (with a median time of exposition of 13.7 years). One of them belonged to the high risk group and 2 to the intermediate risk group. All of them remained in the same SCORE category, despite the adequate control of cardiovascular risk factors.

The Figure 1 shows the distribution of patients according to the cardiovascular SCORE at the time of the first consultation and the last one. We have observed an increased number of patients belonging to the low risk group at the expense of a decrease in those assigned to the intermediate, high, and very high risk groups, with a difference that reaches statistical significance.

The distribution of the patients among the different groups before and after follow-up is low risk (21 *versus* 14 patients), intermediate risk (10 *versus* 14) and high risk (3 *versus* 2). Only one out of three patients remained in the very high risk category.

Regarding arterial thrombosis, one patient receiving treatment with dasatinib presented an episode of acute coronary syndrome. He had a history of hypertension, dyslipidemia and coronary disease prior to TKI initiation, belonging to the high risk SCORE category when starting follow-up.

With respect to the data on thromboembolic disease, only one patient (receiving imatinib as TKI) presented a venous thrombotic event in the form of deep vein thrombosis in the lower limb, arising in the postoperative context of a major abdominal surgery. A patient with a history of antiphospholipid syndrome and deep vein thrombosis prior to the diagnosis of CML received imatinib without new thrombotic events after the start of this drug. No patient treated with second generation TKI or ponatinib developed venous thrombotic events.

At the end of follow-up, 8 patients (22.9%) had been referred to the vascular surgery and angiology consultation. Out of the 8 patients, 7 were referred because of intermittent claudication and 1 for multidisciplinary assessment due to very high cardiovascular risk.

These patients were evaluated with lower limb and carotid doppler. Half of them showed carotid atherosclerosis, but only one presented with significative stenosis (more than 50% of arterial diameter reduction).

Ten patients underwent lower limb doppler in order to rule out significant arterial obstruction. Three patients showed findings of arterial obstruction (those diagnosed with PAOD), four atherosclerotic plaques and three did not reveal pathologic findings.

4 Discussion

In this paper we present the results of cardiovascular control in patients with CML under treatment with TKI in a specific consultation. A reduction in cardiovascular risk factors was achieved with at least a 20% improvement in cardiovascular score.

The baseline characteristics of our cohort are similar to those reported previously in patients with CML: an average age of 57 years and a slight predominance in males (Dahlén et al., 2016). As for the cardiovascular risk factors in our series, the data are comparable to those reported by other authors. The study by Coutinho et al. (Coutinho et al., 2017) showed a rate of hypertension of approximately 30%, like that of our population, and 11% of diabetes (in our study 8.6%). The high proportion of patients with dyslipidemia (40% compared to 18% in the aforementioned study) in our cohort is striking, a difference that may be due to heterogeneity of criteria used to define this condition.

The presence of cardiovascular risk factors or comorbidities is important, on the one hand, for the choice of TKI, given the different toxicity profile of each one, and on the other hand, for the management of such comorbidity (Latagliata et al., 2021). Thus, given that most of our patients received treatment with imatinib and we have a small proportion of patients who received new generation TKIs, it is difficult to make inferences about the relative risk for the development of cardiovascular comorbidity regarding the TKI.

However, according to previous studies, it seems that nilotinib is more associated with the development or worsening of arterial hypertension (Roa-Chamorro et al., 2021), as well as coronary disease together with dasatinib (Barber et al., 2017). Nilotinib is especially associated with stroke (Chen et al., 2021), as well as peripheral arterial disease together with dasatinib (Chen et al., 2021). However, treatment with ponatinib has been the most associated with hypertension (17% vs. 10%) for all new-generation TKI in a pooled analysis of hypertension incidence (Mulas et al., 2021) and thrombotic risk (10% patients developed cerebrovascular or vaso-occlusive disease) (Jain et al., 2015).

For this reason, patient-based therapy has become increasingly important in the treatment of CML (Ciftciler & Haznedaroglu, 2021). The availability of several TKIs has made it possible to choose the most appropriate drug for each patient based on individual factors such as age, comorbidities and availability in each center (Rabian et al., 2019). It

is important to consider factors such as the patient's overall health status, potential side effects, and the risk of developing resistance to the TKI when selecting the best option. In summary, a personalized approach to CML treatment can improve outcomes by maximizing the benefits of therapy while minimizing side effects and reducing risk of treatment resistance (Ciftciler & Haznedaroglu, 2021).

Given that most of our patients received treatment with imatinib in first line and we have a small proportion of patients who received new generation TKIs, it is difficult to make inferences about the relative risk for the development of cardiovascular comorbidity according to the TKI in our CML cohort. Nevertheless, with a median follow-up of 27.8 months, none of the patients who received second generation TKI and who had previous arterial hypertension showed a worsening of this condition (only a patient with imatinib had a deficient control of hypertension during follow-up). 23% of patients were diagnosed of hypertension at some point after TKI initiation. This percentage is slightly lower than that showed by the large cohort of Jain et al. (2019). The only patient who received ponatinib was under antihypertensive treatment before diagnosis of CML and showed an adequate control of hypertension during TKI therapy.

Although the associated thrombotic risk is assessed as a class effect of TKI, the difference in targets of each of the different TKI may explain the differences observed. The Swedish registry showed that patients with CML have an overall risk of venous thromboembolic events and arterial thromboembolic events 1.5 and 2 times higher than general population, respectively (Dahlén et al., 2016). Moreover, second generation TKI and ponatinib seem to confer greater risk than imatinib (Douxfils et al., 2016). In our cohort, the rate of thromboembolic events was low, and these only occurred in patients with strong risk factors. The comparison with other studies is difficult due to difference of median follow-up (Jain et al., 2019). However, these data suggest that follow-up in the specialized consultation may have been effective in preventing thrombotic events.

PAOD rate was surprisingly high in comparison to other cardiovascular events. Other studies show a greater percentage of coronary or cerebrovascular events, with an incidence lower than 1% of PAOD among patients treated with imatinib (Chen et al., 2021). Half of our patients were receiving imatinib at the time of PAOD diagnosis, although nilotinib seems to be more associated with PAOD than other TKI (Douxfils et al., 2016). Yet our patients had a long history of exposition and many cardiovascular risk factors. Our high rate of PAOD could be a consequence of the high suspicion degree maintained in the specific consultation. There are many comorbidities causing lower limbs pain and, unlike cerebrovascular or coronary disease, PAOD is often mildly symptomatic and thus misdiagnosed (Nordanstig et al., 2023). For this reason, nearly one-third of patients underwent a Doppler study and were referred to vascular surgery and angiology consultation.

Another important aspect to consider when controlling cardiovascular risk factors through pharmacological measures is the potential interactions of the TKIs. In our cohort, this had a fundamental impact on the use of statins, as previously seen (Haouala et al., 2011), and for which rosuvastatin or pravastatin are usually recommended, as they are not substrates of CYP3A4 (Osorio et al., 2018).

An appropriate approach to estimating the risk of developing cardiovascular events are prognostic scores, such as the Framingham score, the Pooled Cohort Equations score or the SCORE. Among them, the SCORE model shows many advantages: there are many country-specific versions derived from local data, it is easy to calculate, and it is capable of predicting mortality derived from myocardial infarction, stroke or heart failure over the next 10 years (Caocci et al., 2019).

As results have shown, a significant percentage of patients achieved a change in their risk stratification according to the SCORE, in all cases achieving a better prognosis category than before follow-up, which was achieved thanks to the control of blood pressure, dyslipidemia, or smoking cessation.

Two studies have shown a correlation between the SCORE and the occurrence of cardiovascular events in patients with CML and TKI treatment (although both only included patients with ponatinib) (Breccia et al., 2015; Caocci et al., 2019). Both showed a higher incidence of cardiovascular events in the high and very high risk groups, with a significant difference. In the study by Breccia et al., none of the patients with a low risk SCORE developed cardiovascular disease.

The importance of preventing cardiovascular disease lies in the fact that it is the second leading cause of mortality in cancer patients (Sulpher et al., 2015). For this reason, the importance of a multidisciplinary management of patients with malignant hematological disorders is increasingly been recognized, although we do not find in the literature studies on multidisciplinary management of cardiovascular risk in patients with CML, even when various groups have called attention to this need (García-Gutiérrez et al., 2016; Basile et al., 2022).

Our study shows that this approach to CML patients, in coordination with specialists is feasible and results in an improved control of cardiovascular risk factors. The main limitation of our study is its retrospective nature and the limited number of patients analyzed. In addition, there has been no prolonged follow-up of patients that could demonstrate a reduction in cardiovascular events in patients with a better prognosis SCORE. Among the strengths of the study, it includes patients treated with different TKIs, and the use of a standardized and population-targeted cardiovascular risk model.

5 Conclusion

The adverse effects of tyrosine kinase inhibitors are one of the main concerns when treating patients with chronic myeloid leukemia. These are usually related to their off-target effects and each TKI has a different toxicity profile. Cardiovascular events are among their most frequent and life-threatening complications, and their occurrence can influence the choice or switch of TKI. The development of these events can be prevented by controlling risk factors, which often requires an interdisciplinary management. Our study shows that follow-up in a specialized consultation is an attainable feasible approach that can reduce cardiovascular risk of these patients.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Comité de Ética de la Investigación (CEI) Hospital universitario 12 de Octubre. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CD, DP, JG designed research and collected data, RG and GC-T interpreted the data and performed statistical analysis, AB and RG wrote the manuscript, CD, RA, GC-T, and JM-L designed and supervised the overall study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Olverembatinib (HQP1351)-based therapy in adults with relapsed or refractory Philadelphia chromosome-positive acute lymphoblastic leukemia or chronic myeloid leukemia in blast phase: results from a real-world study

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Background: Relapsed or refractory Philadelphia chromosome-positive acute lymphoblastic leukemia (R/R Ph^+ ALL) and chronic myeloid leukemia in the blast phase (CML-BP) are associated with poor prognoses. Olverembatinib (HQP1351), a novel third-generation tyrosine kinase inhibitor (TKI), has shown promising efficacy and safety in clinical trials against nearly all BCR-ABL1 kinase mutations, including *T315I*.

Methods: Data were collected and analyzed to evaluate the efficacy and safety of olverembatinib-based therapy for advanced Ph^+ leukemia. The primary outcome was the overall response rate at 28 days. Secondary outcomes included overall survival (OS), event-free survival (EFS), disease-free survival (DFS), the proportion of patients undergoing allo-HSCT, and adverse events.

Results: A total of 59 patients participated in the study, including 40 patients with Ph^+ ALL and 19 with CML-BP. Among them, 36 (61.0%) were men, and 23 (39.0%) were women. The median age was 39 years (interquartile range [IQR], 30–48), and the median follow-up duration was 7.8 months (IQR, 4.1–11.3). A total of 16 (27.1%) and 11 patients (18.6%) had received treatment with two and ≥ 3 prior TKIs, respectively. Additionally, 19 patients (33.9%) had been treated with ponatinib. In a cohort of 19 CML patients, 12 (63.2%) achieved CR/CRi by day 28. Five (26.3%) achieved a complete cytogenetic response with a median duration of 2.9 months, and two (10.5%) achieved a major molecular response with a median duration of 5.5 months. Among 40 evaluated ALL patients, 37 (92.5%) achieved CR/CRi by day 28, 30 (75.0%) attained MRD negativity, and 22 (55.0%) achieved CMR. The probabilities of DFS, EFS, and OS at 12 months were 80.3% (95% confidence interval [CI]: 61.0%–90.7%), 80.2% (95% CI: 61.0%–90.7%), and 93.3% (95% CI:

75.8%–98.3%) for patients with R/R Ph$^+$ ALL, compared to 52.0% (95% CI: 17.7%–78.0%), 23.0% (95%CI, 4.2%-50.6%), and 75.6% (95% CI: 37.7%–92.3%) for those with CML-BP. The prevalent treatment-related nonhematologic adverse events, primarily classified as grade 1/2, included skin hyperpigmentation, proteinuria, increased liver enzyme levels, and hypertriglyceridemia.

Conclusions: Olverembatinib-based therapy demonstrated significant efficacy and manageable toxicity in patients with advanced Ph$^+$ leukemia.

KEYWORDS

acute lymphoid leukemia, Philadelphia chromosome positive, Ph$^+$, chronic myeloid leukemia, blast phase, olverembatinib, HQP1351

1 Introduction

Relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemia (R/R Ph$^+$ ALL) and chronic myeloid leukemia (CML) in blast phase (BP) are aggressive leukemias with a poor prognosis (1–4). Tyrosine kinase inhibitors have significantly improved survival and quality of life for patients with Ph$^+$ leukemia, particularly third-generation tyrosine kinase inhibitors (TKIs) for those harboring various *BCR::ABL1* mutations (5–8).

Olverembatinib is a novel inhibitor that targets the ATP-binding site of the *BCR::ABL1* kinase. The National Medical Products Administration approved its use in November 2021 for adult chronic or accelerated-phase CML patients harboring the *T315I* mutation. In November 2023, it received approval for a new indication in adult patients with chronic-phase CML who are resistant and/or intolerant to first- and second-generation TKIs (9). Recently, olverembatinib was approved to initiate a global phase III clinical trial and has been included in the latest version of the National Comprehensive Cancer Network guidelines for the treatment of CML (10). The complete remission rate exceeds 90% when frontline treatment for newly diagnosed Ph+ ALL incorporates TKIs in combination with chemotherapy or immunotherapy. However, relapse remains a significant clinical challenge, frequently associated with resistance mutations in the ABL1 kinase domain, particularly the T315I mutation (11–13).

The efficacy and toxicity of an olverembatinib-based regimen in Ph$^+$ advanced leukemia remain unclear. Currently, no unified guidelines exist for its treatment strategy. Therefore, we conducted this study to evaluate the activity and safety of olverembatinib in Ph$^+$ advanced leukemia.

2 Methods

2.1 Study design and patients

This retrospective multicenter study was conducted at three hospitals in China—Nanfang Hospital, Guangdong Second Provincial General Hospital, and The Second Affiliated Hospital of Guangzhou Medical University—from December 2021 to October 2023. Data were retrospectively collected and analyzed data from patients aged 14 to 70 years with an Eastern Cooperative Oncology Group performance status of 0 to 2, diagnosed with R/R Ph$^+$ ALL or advanced CML according to World Health Organization 2022 classification (14) and the 2020 European LeukemiaNet criteria (15). Exclusion criteria included impaired cardiac function, severe cardiovascular disease, and lactating or pregnant women.

2.2 Treatment

Drug doses were administered per prescribed instructions and institutional protocols. Olverembatinib was given at 40 mg orally with meals every 2 days within a 28-day dosing cycle. Treatment continued until disease progression, intolerable toxicity, or other circumstances required cessation of treatment.

Patients diagnosed with myeloid blast phase (MBP) could receive olverembatinib either as monotherapy or in combination with systemic chemotherapy or blinatumomab. Chemotherapy regimens included the HA regimen (homoharringtonine and cytarabine) and hypomethylating agents (HMA) such as azacitidine or decitabine. Patients with lymphoid blast phase (LBP) were treated with either monotherapy or a combination therapy involving the vincristine, daunorubicin, and prednisone (VDP) regimen or blinatumomab. Patients with Ph$^+$ ALL could receive olverembatinib in combination with blinatumomab, systemic chemotherapy, or radiotherapy (for extramedullary leukemia only). Chemotherapy regimens included the VDP regimen and the hyper-fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (hyper-CVAD) regimen. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) was considered a potential option after achieving a response if a patient was eligible and a donor was available. Routine intrathecal injection (dexamethasone, 10 mg; cytarabine, 50 mg; and methotrexate, 10 mg) was administered to prevent or treat central nervous system leukemia (16, 17). Eligible patients underwent

comprehensive baseline assessments, including a detailed medical history review, physical examination, imaging tests, and laboratory analyses, which covered peripheral blood assessments, bone marrow evaluations, and *BCR::ABL1* transcript analysis.

2.3 Assessments

Several outcomes were evaluated, including response rates, overall survival (OS), event-free survival (EFS), disease-free survival (DFS), the proportion of patients undergoing allo-HSCT, and the toxicity of olverembatinib. Complete response (CR) and complete response with incomplete hematological recovery (CRi) were defined as bone marrow blasts < 5% and no extramedullary disease, with or without neutrophil counts < 1.0×10^9/L and platelet counts < 100×10^9/L. OS was defined as the time from the initiation of olverembatinib to death from any cause. EFS was defined as the time from the initiation of olverembatinib to the earliest occurrence of any of the following events: failure at day 28, relapse, treatment discontinuation, or death. DFS was defined as the time from CR/CRi to relapse or death. *BCR::ABL1* transcripts in bone marrow aspirates (when available) or peripheral blood were detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), with results expressed on the International Scale (IS). Major molecular response (MMR) and complete molecular response (CMR) were defined as a *BCR::ABL1* transcript level of ≤ 0.1% and undetectable or below the 10^{-5} level by RT-qPCR, respectively. A BCR::ABL1 transcript level of ≤ 1% was considered equivalent to complete cytogenetic remission (CCyR). Measurable residual disease (MRD) negativity was commonly defined using a cutoff of 0.01% by eight-color flow cytometry (18, 19). Additionally, Sanger sequencing was performed in CML patients to assess *BCR::ABL1* mutational status. Adverse events were documented and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0.

2.4 Statistical analysis

Descriptive analysis will be used for baseline data and adverse events, reporting numbers and frequencies for qualitative data and medians with interquartile ranges (IQRs) for quantitative data. Kaplan–Meier survival curves were generated for OS, EFS, and DFS. All statistical analyses in this study were conducted using SPSS version 25.0 and GraphPad Prism.

3 Patient characteristics

Between 8 December 2021 and 22 October 2023, a total of 59 patients who received olverembatinib-based therapy were included in the study. Among them, 19 patients were diagnosed with CML-BP, including 11 with MBP, seven with LBP, and one with mixed blast phase (MAL), exhibiting both myeloid and lymphoid features. Most patients had previously received TKI treatments, including imatinib, dasatinib, nilotinib, flumatinib, and ponatinib. Patient characteristics are summarized in Table 1.

3.1 CML patients

Among the 19 patients, 14 (73.7%) were men and five (26.3%) were women. The median age was 45 years (IQR: 33–48); the median time from diagnosis to the initiation of olverembatinib was 20.1 months (IQR: 10.2–87.5); and the median follow-up duration was 5.6 months (IQR: 4.1–11.9). All patients exhibited the p210 type of *BCR::ABL1* transcripts. A total of 47.4% (9/19) had received ≥ 3 prior TKIs, while 42.1% (8/19) had received two prior TKIs. Additionally, 47.4% (9/19) had been pretreated with ponatinib, among whom 66.7% developed drug resistance and 33.3% discontinued due to other reasons. Genetic testing revealed that 73.7% (14/19) of patients had mutations. The most prevalent mutations within the *ABL1* kinase domain were *T315I* (42.1%) and *E255K* (15.8%), along with other mutations such as *E255V*, *K357E*, *E462G*, *P1108P*, *D198G*, *M293*, and *H396*. The most common *non-ABL1* kinase domain mutations were *RUNX1* (26.3%) and *ASXL1* (26.3%). Additionally, three (15.8%) patients presented with central nervous system leukemia (CNSL), one (5.3%) had extramedullary lymph node leukemia, and one (5.3%) had undergone allo-HSCT before the initiation of the study.

3.2 ALL patients

Among the 40 patients, 22 (55.0%) were men and 18 (45.0%) were women. The median age was 38 years (IQR: 28–48); the median time from diagnosis to initiation of olverembatinib was 8.2 months (IQR: 2.6–10.8); and the median follow-up was 8.3 months (IQR: 5.5–12.5). Patients had either the p210 (13/40; 32.5%) or p190 (27/40; 67.5%) form of *BCR::ABL1* transcripts. A total of eight patients (20.0%) had been treated with two prior TKIs, while two (5.0%) had received ≥ 3. Additionally, 10 patients (25.0%) had been treated with ponatinib, of whom 50.0% developed resistance, 20.0% experienced intolerance, and 30.0% discontinued for other reasons. Three patients had mutations in the *ABL1* kinase domain, including *T315I* (2.5%), *E255K* (2.5%) and *F317L* (2.5%). A total of nine patients (22.5%) had CNSL. Among the 40 patients with Ph^+ ALL, 26 (65.0%) had primary refractory ALL, while 14 (35.0%) had relapsed refractory ALL, of whom 11 (78.6%) had previously undergone hematopoietic stem cell transplantation.

4 Outcomes

Tables 2–4 summarize patient disposition, treatment regimens, and treatment outcomes.

TABLE 1 Patient characteristics.

	Total	Ph^+ ALL	CML-BP
Patient number	59	40	19
Age (year; median (IQR))	39 (30–48)	38 (28–48)	45 (33–48)
Male (*n* (%))	36 (61.0)	22 (55.0)	14 (73.7)
Female (*n* (%))	23 (39.0)	18 (45.0)	5 (26.3)
ECOG performance status (*n* (%))			
≤ 1	56 (94.9)	38 (95.0)	18 (94.7)
=2	3 (5.1)	2 (5.0)	1 (5.3)
Time from diagnosis to olverembatinib treatment (month; median (IQR))	6.3 (2.5–20.1)	8.2 (2.6-10.8)	20.1 (10.2–87.5)
BCR::ABL1 transcript (*n* (%))			
p210	32 (54.2)	13 (32.5)	19 (100)
p190	27 (45.8)	27 (67.5)	0 (0)
Number of lines of prior TKI therapy (*n* (%))			
0	10 (16.9)	10 (25.0)*	0 (0)
1	22 (37.3)	20 (50.0)	2 (10.5)
2	16 (27.1)	8 (20.0)	8 (42.1)
3	7 (11.9)	1 (2.5)	6 (31.6)
4	4 (6.8)	1 (2.5)	3 (15.8)
Type of prior TKI therapy (n (%))			
Imatinib	12 (20.3)	3 (7.5)	9 (47.4)
Nilotinib	10 (16.9)	1 (2.5)	9 (47.4)
Dasatinib	41 (69.5)	26 (65.0)	15 (78.9)
Flumatinib	9 (15.3)	3 (7.5)	6 (31.6)
Ponatinib	19 (33.9)	10 (25.0)	9 (47.4)
BCR::ABL1 mutation status (n (%))			
Non-T315I BCR::ABL1 mutation	8 (13.6)	2 (5.0)	6 (31.6)
T315I mutation	9 (15.3)	1 (2.5)	8 (42.1)
Receive HSCT before olverembatinib *n* (%)	12 (20.3)	11 (27.5)	1 (5.3)
With CNSL (n (%))	12 (20.3)	9 (22.5)	3 (15.8)

IQR, interquartile range; ECOG, Eastern Cooperative Oncology Group; TKI, tyrosine kinase inhibitors; HSCT, hematopoietic stem cell transplant; CNSL, central nervous system leukemia. *Ten patients without TKI maintenance therapy post-HSCT experienced disease relapse (Pre-HSCT: 3 imatinib, 7 dasatinib).

TABLE 2 Patient disposition.

	Total	Ph^+ ALL	CML-BP
Patient number	59	40	19
Treatment duration month; median (IQR)	7.8 (4.1–11.3)	8.3 (5.5–12.5)	5.6 (4.1–11.9)
Ongoing *n* (%)	37 (62.7)	29 (72.5)	8 (42.1)
Discontinuation *n* (%)	22 (37.3)	11 (27.5)	11 (57.9)
Adverse events	0	0	0
Treatment failure	6 (10.2)	4 (10.0)	2 (10.5)
Death	5 (8.5)	2 (5.0)	3 (15.8)
Other reasons unrelated to efficacy	11 (18.6)	5 (12.5)	6 (31.6)
CR/CRi at day 28 *n* (%)	49 (83.1)	37 (92.5)	12 (63.2)
CR/CRi of pre-ponatinib *n* (%)	14 (73.7)	9 (90.0)	5 (55.6)
CR/CRi, *T315I*-mutated population *n* (%)	5 (55.6)	1 (100.0)	4 (50.0)
MRD negative by FCM	–	30 (75.0)	–
Receive HSCT after CR/CRi *n* (%)	18 (30.5)	13 (32.5)	5 (26.3)
Receive CAR-T after CR/CRi *n* (%)	3 (5.1)	3 (7.5)	0
Receive DLI after CR/CRi *n* (%)	3 (5.1)	3 (7.5)	0

IQR, interquartile range; CR, complete response; CRi, complete response with incomplete hematological recovery; CR/CRi of pre-ponatinib, CR/CRi of patients who had prior ponatinib treatment; MRD, measurable residual disease; HSCT, hematopoietic stem cell transplant; CAR-T, chimeric antigen receptor T cell; DLI, donor lymphocyte infusion.

4.1 CML patients

In a cohort of 19 patients with CML-BP, 12 (63.2%) achieved CR/CRi by day 28. Among them, two (10.5%) attained MMR with a median duration of 5.5 months, and five (26.3%) achieved CCyR with a median duration of 2.9 months. Five patients received olverembatinib alone, two were treated in combination with hypomethylating agents, 11 underwent systemic chemotherapy in combination, and one received olverembatinib with blinatumomab. Their CR/CRi rates after 28 days of treatment were 60.0%, 0%, 72.7%, and 100.0%, respectively. All patients who had prior ponatinib treatment received olverembatinib in combination with chemotherapy, achieving a CR/Cri rate of 55.6%. Only one ponatinib-resistant patient attained MMR. A total of five (26.3%) patients underwent allo-HSCT, including one individual with *BCR::ABL*1 transcript levels (IS) > 10%, who experienced relapse within 3 months posttransplantation. The 12-month probabilities of OS, EFS, and DFS in patients with CML-BP were 75.6% (95% confidence interval (CI): 37.7%–92.3%), 23.0% (95%CI, 4.2%-50.6%), and 52.0% (95% CI, 17.7%–78.0%), respectively (Figure 1).

4.2 ALL patients

In a cohort of 40 patients with Ph^+ ALL, 37 (92.5%) achieved CR/CRi by day 28, and 30 (75.0%) attained MRD negativity. Additionally, 22 (55.0%), including one patient with the *T315I* mutation, reached CMR. Of these, eight patients received combination therapy with blinatumomab, while 31 underwent

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TABLE 3 Response of patients with CML-BP to olverembatinib alone or in combination with other drugs.

CML-BP	LBP (*n* = 7)	MBP (*n* = 11)	MAL (*n* = 1)	CR/CRi (*n* (%))
HMA	0	2	0	0
HA	0	6	1	5 (71.4)
VDP	4	0	0	3 (75.0)
Blinatumomab	1	0	0	1 (100.0)
Olverembatinib only	2	3	0	3 (60.0)

combination therapy with chemotherapy, with CR/CRi rates of 87.5% and 93.5%, respectively. Among ponatinib-pretreated patients, seven received combination therapy with chemotherapy, and three received combination therapy with blinatumomab. In this subgroup, 90.0% achieved CR/Cri, and 80.0% attained MMR. After achieving CR/CRi, 13 (32.5%) patients proceeded to undergo allo-HSCT, while three (7.5%) patients with a history of previous transplantation received donor lymphocyte infusion (DLI). Chimeric antigen receptor T-cell (CAR-T) therapy was administered to three patients with BCR::ABL1 transcripts (IS) > 10%; however, one patient experienced relapse 4 months later. The 12-month probabilities of OS, EFS, and DFS in Ph^+ ALL patients were 93.3% (95% CI: 75.8%–98.3%), 80.2% (95% CI: 61.0%–90.7%), and 80.3% (95% CI: 61.0%–90.7%), respectively (Figure 1).

5 Safety

Since systemic chemotherapy and immunotherapy can cause myelosuppression, treatment-related hematological adverse events were not documented. With a median follow-up of 7.8 months after olverembatinib initiation, the incidence of adverse events was comparable between the ALL and CML cohorts (Table 5). The most commonly reported nonhematologic adverse events were mild (grade 1/2) and resolved either with dosage reduction, supportive therapy, or spontaneously without intervention. Nonhematologic adverse events included skin pigmentation (*n* = 38), proteinuria (*n* = 32), elevated liver enzyme levels (*n* = 25), hypertriglyceridemia (*n* = 24), hyperuricemia (*n* = 8), hyperbilirubinemia (*n* = 8), rash (*n* = 6), arterial occlusion events (*n* = 2), and elevated creatine kinase (*n* = 1).

In the study, no patients experienced treatment-related pancreatitis, hypertension, arrhythmia, prolonged QT interval, pleural effusion, pericardial effusion, or gastrointestinal adverse events such as diarrhea and constipation. Among all patients, 37 (62.7%) continued treatment, while 22 (37.3%) discontinued due to disease progression, intolerance, loss to follow-up, death, or other reasons.

6 Discussion

This study demonstrated that olverembatinib-based therapy exhibited significant efficacy in heavily pretreated patients with advanced Ph^+ leukemia who had developed resistance to multiple TKIs.

The prognosis for newly diagnosed CML-BP patients or those progressing from CP/AP to BP is notably poor, with a median overall survival of less than 1-year postdiagnosis (20–23). Third-generation TKIs have demonstrated the ability to achieve deeper responses than first- and second-generation TKIs and effectively overcome resistance (9, 24–26). Olverembatinib is the only third-generation TKI available in China. However, research on the efficacy of olverembatinib-based therapy in advanced CML remains limited. Jiang et al. reported that among 38 patients with TKI-resistant CML-AP treated with olverembatinib, 18 achieved MCyR and CCyR at a median time of 3 months (range, 1–9) and 4 months (range, 1–15), respectively. The rates of MMR and MR4.0 were 44.7% and 36.8%, respectively (24). In this study, 12 (63.2%) patients with CML-BP achieved CR/CRi by day 28. Additionally, two (10.5%) reached MMR at a median time of 5.5 months, while five (26.3%) reached CCyR at a median time of 2.9 months. The 12-month probabilities of OS, EFS, and DFS in patients with CML-BP were 75.6% (95% CI: 37.7%–92.3%), 23.0% (95%CI, 4.2%-50.6%) and 52.0% (95% CI: 17.7%–78.0%), respectively. Despite nearly half of the CML patients in our study having prior treatment with ponatinib and 3 (15.8%) presenting with CNSL, our outcomes closely aligned with those reported in the studies by Jiang et al. and the PACE trial (24, 26). These findings suggest that

TABLE 4 Response of patients with Ph^+ ALL to olverembatinib alone or in combination with other drugs.

Ph^+ ALL	Primary refractory ALL (*n* = 26)	Relapse with CNSL (*n* = 9)	Relapse without CNSL (*n* = 5)	CR/CRi (*n* (%))
VDP ± venclexta	15	5	2	21 (95.5)
Hyper-CVAD	6	2	1	8 (88.9)
Blinatumomab	5	1	2	7 (87.5)
Radiotherapy	0	1	0	1 (100.0)

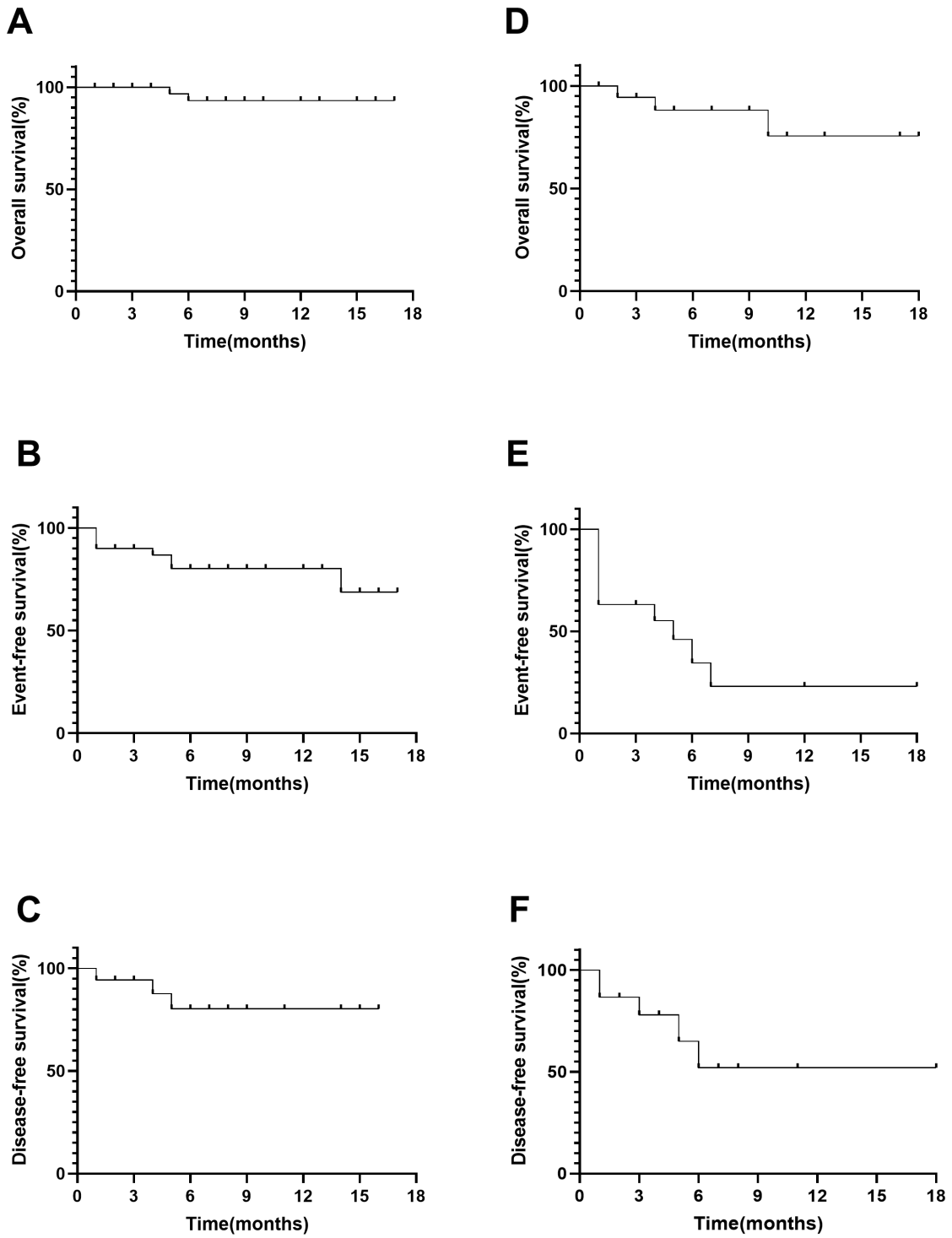

FIGURE 1
Overall survival, event-free survival, and disease-free survival in ALL **(A–C)** and CML **(D–F)**.

olverembatinib-based treatment is a potent and effective induction therapy for patients with CML-BP.

Olverembatinib more effectively inhibits p-KIT, p-AKT, p-ERK1/2, and p-STAT3 than ponatinib, targeting markers highly expressed in hematological malignancies. It suppresses pre-B ALL cells by inhibiting SRC kinase and the PI3K/AKT pathways, highlighting its potential as a therapeutic agent for pre-B ALL (27–29). A recent study by Jabbour et al. (25) demonstrated the promising efficacy of olverembatinib in patients with heavily pretreated or refractory CML and Ph^+ ALL outside of China. In the evaluable cohort of ponatinib-failed patients, 53.3% attained CCyR and 37.5% achieved MMR in the CML-CP group, while

TABLE 5 Treatment-related adverse events.

No. (%)	Total (n = 59)		ALL (N = 40)		CML (N = 19)	
Nonhematological AEs	Any grade	Grade 3/4	Any grade	Grade 3/4	Any grade	Grade 3/4
Skin pigmentation	38 (64.4)	0	30 (75.0)	0	8 (42.1)	0
Proteinuria	32 (54.2)	0	24 (60.0)	0	8 (42.1)	0
Elevated liver enzyme	25 (42.4)	4 (6.8)	18 (45.0)	3 (7.5)	7 (36.8)	1 (5.3)
Hypertriglyceridemia	24 (40.7)	2 (3.4)	21 (52.5)	2 (5.0)	3 (15.8)	0
Hyperuricemia	8 (13.6)	1 (1.7)	7 (17.5)	1 (2.5)	1 (5.3)	0
Hyperbilirubinemia	8 (13.6)	1 (1.7)	5 (12.5)	1 (2.5)	3 (15.8)	0
Rash	6 (10.2)	0	5 (12.5)	0	1 (5.3)	0
Arterial occlusion event	2 (3.4)	1 (1.7)	2 (5.0)	1 (2.5)	0	0
Elevated creatine kinase	1 (1.7)	1 (1.7)	0	0	1 (5.3)	1 (5.3)

Treatment-related hematological adverse events were not documented.

28.6% attained CCyR and 22.2% achieved MMR in the ALL group. In our study, among ponatinib-pretreated patients, the rates of MMR were 80.0% in the ALL cohort and 11.1% in the CML-BP cohort. Compared to the study by Jabbour et al., outcomes in the ALL cohort appear more favorable, whereas those in the CML cohort are less satisfactory. One possible explanation for this discrepancy is that most Ph^+ ALL patients who had failed third-generation TKI therapy in Jabbour's study were treated exclusively with olverembatinib, whereas the CML patients included were in the chronic phase. Collectively, these findings support the potential of olverembatinib as a highly effective therapeutic backbone agent for patients with Ph^+ ALL resistant to multiple TKIs.

Xiang et al. successfully detected olverembatinib concentrations in the patient's cerebrospinal fluid (CSF) (30). Li et al. also confirmed that in pediatric patients with relapsed refractory ALL and CNSL, the addition of olverembatinib can rapidly eliminate leukemia blast cells in CSF (31). In this study, eight of 12 patients with CNSL achieved CSF negativity following olverembatinib-based treatments, further suggesting a potential role for olverembatinib in managing CNSL in patients with Ph^+ leukemia.

Common AEs in patients receiving olverembatinib therapy are predominantly hematological, dermatological, or associated with proteinuria and abnormal biochemical indicators (9, 24). The most common nonhematological AEs were elevated liver enzymes, proteinuria and skin pigmentation. Jiang et al. reported a 32.0% incidence of cardiovascular events related to olverembatinib during a median follow-up of 3 years, with arterial occlusive and venous thrombotic events occurring in 5.0% of patients—lower than the 31.0% reported for ponatinib over a 5-year median follow-up (24). This study reported that two patients (3.4%) experienced arterial occlusion events, including cerebral infarction and pulmonary embolism. Of note, both patients had prior exposure to ponatinib. Nonetheless, vigilant monitoring remains essential for cardiovascular thrombotic events associated with olverembatinib.

This study has several limitations: the heterogeneity of patient populations and treatment regimens limits the ability to draw general conclusions; its retrospective nature precludes a detailed assessment of treatment-related adverse events; and the small sample size hinders a comprehensive evaluation of the cardiovascular toxicity of olverembatinib. However, a key strength of observational studies is their relevance to real-world patient populations.

7 Conclusion

In summary, these findings demonstrate that the olverembatinib-based regimen shows favorable efficacy and safety in patients with CML-BP and Ph^+ ALL. Prospective randomized controlled clinical trials with larger and more diverse patient populations are needed to confirm these results.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Nanfang Hospital, Southern Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because This study was a retrospective study that used archived medical records and data, did not disclose subjects' personal information, and did not cause any risks or harm to the subjects. The use of anonymous data not only protected personal privacy but also enabled valuable analysis.

Author contributions

ZW: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. ZL: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. XY: Writing – review & editing. ZF: Resources, Writing – review & editing. RL: Resources, Writing – review & editing. FH: Resources, Writing – review & editing. LX: Resources, Writing – review & editing. XFL: Resources, Writing – review & editing. HJ: Resources, Writing – review & editing. MD: Resources, Writing – review & editing. JS: Resources, Writing – review & editing. XZ: Resources, Writing – review & editing. QW: Resources, Writing – review & editing. XL: Resources, Writing – review & editing. QL: Conceptualization, Methodology, Project administration, Writing – review & editing. HZ: Conceptualization, Methodology, Project administration, Writing – review & editing. NX: Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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