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Nilotinib-induced bone marrow CD34+/lin-Ph+ cells early clearance in newly diagnosed CP-Chronic Myeloid Leukemia: Final report of the PhilosoPhi34 study

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Abstract

Chronic Myeloid Leukemia is a clonal disorder characterized by the presence of the Ph-chromosome and the BCR-ABL tyrosine-kinase (TK). Target-therapy with Imatinib has greatly improved its outcome. Deeper and faster responses are reported with the second-generation TKI Nilotinib. Sustained responses may enable TKI discontinuation. However, even in a complete molecular response, some patients experience disease recurrence possibly due to persistence of quiescent leukemic CD34+/lin−Ph+ stem cells (LSCs). Degree and mechanisms of LSCs clearance during TKI treatment

are not clearly established. The PhilosoPhi34 study was designed to verify the in-vivo activity and timecourse of first-line Nilotinib therapy on BM CD34+/lin−Ph+ cells clearance. Eighty-seven CP-CML patients were enrolled. BM cells were collected and tested for Ph+ residual cells, at diagnosis, 3, 6 and 12 months of treatment. FISH analysis of unstimulated CD34+/lin− cells in CCyR patients were positive in 8/65 (12.3%), 5/71 (7%), 0/69 (0%) evaluable tests, respectively. Per-Protocol analysis response rates were as follows: CCyR 95% at 12 months, MR4.5 31% and 46% at 12 and 36 months, respectively. An exploratory Gene Expression Profiling (GEP) study of CD34+/lin− cells was performed on 30 patients at diagnosis and after, on 79 patients at diagnosis vs 12 months of nilotinib treatment vs 10 healthy subjects. Data demonstrated some genes significantly different expressed: NFKBIA, many cell cycle genes, ABC transporters, JAK-STAT signaling pathway (JAK2). In addition, a correlation between different expression of some genes (JAK2, OLFM4, ICAM1, NFKBIA) among patients at diagnosis and their achievement of an early and deeper MR was observed.

KEYWORDS

Chronic Myeloid Leukemia, GEP, NFKBIA, nilotinib, stem cells

1 | INTRODUCTION

Chronic Myeloid Leukemia (CML) is the first disease identified by a molecular marker and the first in which the targeted therapy totally modified the natural history of disease.

The reciprocal translocation t(9;22) (q34;q11) results in the constitutively activated fusion gene that encodes for a BCR-ABL tyrosine kinase[1] and it is detected in all patients. The BCR-ABL tyrosine kinase activity imparts growth advantage to leukemic cells, increases proliferation and the cytokine-independent growth, inhibits apoptosis, and alters adhesion pathways[2-5]. Until now, no clear data are available about the in vivo impact between the different genes/pathways expression and the CML response to treatment.

CML can be effectively treated with the first-line tyrosine kinase inhibitor (TKI) Imatinib to obtain a high cumulative best complete cytogenetic response (CCyR) rate[6] and a high estimated event-free survival.[7] An independent study on 832 CML patients enrolled in CCyR after 2 years of Imatinib treatment showed an overall survival of 95% that is similar to that of the general population.[8]

Moreover, some studies[9-11] suggested that Imatinib can be safely discontinued in patients with a complete molecular response; however, approximately 50% of patients relapsed within 6 months of treatment discontinuation. Relapse after Imatinib discontinuation could be traced back to the variable persistence of CD34+ Ph+ quiescent stem cells even in optimal responders[12].

Compared to Imatinib, deeper and faster molecular responses can be obtained in CML patients with the second-generation TKIs Nilotinib and Dasatinib.

Nilotinib is a potent, selective inhibitor of BCR-ABL, rationally designed to improve target specificity over Imatinib.

It is approved for first-line treatment of patients with Ph+ CP-CML based on the results of the ENESTnd study[13-16] which showed superior molecular responses (MMR, MR4, and MR4.5) and significantly lower rates of progression to accelerated phase (AP) or blastic crisis (BC). Moreover, for the first time, this study demonstrated an association between deeper and faster responses and progression prevention. When sustained, these responses may enable more patients to attempt TKI discontinuation[17,18].

Key Points

- Nilotinib 300 mg BID rapidly affects BM CD34+/lin-Ph+ cells, probably due to the involvement of cell mediators other than BCR-ABL.
- The *NFKBIA* down-regulation at diagnosis seems to correlate with a significantly deeper molecular response during Nilotinib treatment.
- What is the new aspect of your work?

Our manuscript shows the clearance of the bone marrow CD34+/lin-Ph+ stem cells of CML patients during Nilotinib treatment in a large prospective study. In addition, we report some gene expression profiling analyses in this prospective study.

- What is the central finding of your work?

In this paper, we integrate clinical results with gene expression profiling data and FISH analyses. We have identified some subgroups of patients with a major probability to obtain an early and deeper response.

- What is (or could be) the specific clinical relevance of your work?

These data allow us to speculate new therapeutic strategies for those patients who cannot obtain an optimal response/a treatment-free remission.

Despite the deeper and faster responses induced by Nilotinib in a large proportion of patients, the eradication of the pathological stem cells has not yet been confirmed, and in vitro data suggest that quiescent stem cells are indeed not sensitive to BCR/ABL inhibition[19,20].

In this regard, during Imatinib treatment, the persistence of residual CD34+Ph+ progenitor cells has been documented in vivo, even in patients with prolonged CCyR and MMR; Bocchia et al[12] demonstrated that approximately 45% of patients still harbored a median of 1% (range, 1%-7%) CD34+ Ph+ CML cells in the BM.

A preliminary study by Defina et al, 2012,[21] found that, compared to Imatinib-treated patients in CCyR, residual leukemic progenitors were very rarely detected in Nilotinib-treated patients who obtained CCyR even after shorter median therapy duration (39 vs 22 months). Specifically, this study demonstrated undetectable CD34+Ph+ cells after only 3 months of Nilotinib treatment in five patients in CCyR.

Despite the very limited number of the five patients reported, these in vivo data suggest that the rapid inhibitory activity of Nilotinib on CML burden may affect stem cells as well.

This is in apparent contrast with the in vitro evidence suggesting no superiority of Nilotinib compared with Imatinib in inducing growth suppression of CML progenitor cells [22]. Therefore, additional data and longer follow-up are required to clarify whether:

1. Nilotinib is truly more efficient than Imatinib in eliminating CML quiescent stem cells in vivo;
2. This will translate into a significantly higher number of CMR patients eligible for treatment discontinuation with a good expectancy of prolonged treatment-free remission (TFR).

The aim of this study was to establish the clearance of Ph+ stem cells pool (CD34+/lin−) in BM during first-line Nilotinib 300 mg BID treatment. Secondary endpoints included outcome data at different time points.

1.1 | Exploratory study

The PhilosoPhi34 study also included an exploratory study aimed to evaluate GEP of selected CD34+/lin− cells of CML patients at diagnosis and at 12 months of treatment as well as 10 healthy donors (CTRLs) using Affymetrix GeneChip Instruments and Software Systems, and Affymetrix GeneChip Human Genome HTA 2.0 (whole human transcriptome analysis).

2 | METHODS

2.1 | Study design

The PhilosoPhi34 study (EudraCT: 2012-005062-34) is a multicenter, prospective, single-arm study in newly diagnosed CP-CML patients treated with Nilotinib 300 mg BID. It has been designed by the Rete Ematologica Lombarda (REL), a network which includes hematology centers in the Lombardia region in Northern Italy. The Coordinating Centre is the Niguarda Hospital in Milano.

This study is divided into three consecutive phases: an initial recruitment phase, a "core" phase of treatment lasting for 12 months, and an "observational" phase lasting for an additional period of 24 months starting on the day of last drug dose administration during the core phase.

2.2 | Study endpoints

The primary efficacy endpoint was the rate of residual CD34+/lin-Ph+ cells in the BM of CCyR patients after 6 months of Nilotinib treatment, using cell selection system and FISH analysis.

Secondary endpoints estimated: (a) the clearance of CD34+/lin-Ph+ cells in the BM of CCyR patients at 3 and 12 months of treatment; (b) the CCyR rate at 3, 6, and 12 months of treatment; (c) the percentage of patients with MR ≤ 10% IS and MR ≤ 1% IS in the peripheral blood (PB) at 3 and 6 months, respectively; (d) the major molecular response (MMR) IS and the MR 4,5 IS in the PB by 3-, 6-, and 12-month periods, respectively.

2.3 | Study population

The study included adult (≥18 years), male or female newly diagnosed Ph+ CP-CML patients; Ph- patients or patients with variant translocations by standard cytogenetic analysis but Ph+ by FISH were also eligible. Additional inclusion and exclusion criteria are defined as we have previously reported[23].

2.4 | Treatment schedule

Nilotinib 300 mg BID standard dose was administered for one year (core phase). At the end of this core phase of the trial, two additional years of observation were planned. During the observational period, treatment options were at the Investigator's choice (ie, any TKI approved as first-line treatment could be used), based on the individual patient evaluation.

Since Nilotinib 300 mg BID has already been registered as first-line treatment of CP-CML, TKI toxicity control and dose adjustments during the study were managed according to the prescribing information of the manufacturer.

2.5 | Evaluation of response to treatment

Patient's response to treatment was monitored according to ELN recommendations[24,25]. Briefly, cytogenetic analyses were performed at 3, 6, and 12 months at local REL laboratories; the molecular response was evaluated on PB cell samples at 3 months intervals, and analyses were performed at the local LabNet-standardized laboratories (IS)[26,27].

2.6 | Study analyses

2.6.1 | Collection and selection of BM CD34+/lin− cells

BM blood samples of all enrolled patients were collected at diagnosis and after 3, 6, and 12 months of treatment. In addition, we collected BM blood samples of 10 healthy donors. BM mononuclear cells (BMMNCs) from all samples of CML patients and healthy donors were isolated, and immediately afterward, we selected BM CD34+/lin− cells (ie CD34+CD38+/CD38− cells) using Diamond CD34 Isolation kit and autoMACs Pro separator (Miltenyi Biotec). The methods were described in http://dx.doi.org/10.17504/protocols.io.yncfvaw and showed in our previous study. The selection method isolated all the CD34+/lin− cells from CML patients which might include healthy stem cells beyond CML stem cells. The purity of BM CD34+/lin− cell samples was determined by flow cytometry analysis.

2.6.2 | FISH analysis on CD34+/lin− cells

The standard FISH analysis was performed on isolated, unstimulated CD34+/lin− BM cells by the Cytogenetic Laboratory of the coordinating center. For each patient, a sample containing at least 10^3 of selected cells fixed in Carnoy's solution was analyzed. Considering the low sensitivity of the test, at least 200 nuclei were examined in order to define the test as negative; conversely, all positive tests were accepted as evaluable, aside from the number of nuclei available for analysis.

2.6.3 | GEP experiments and bioinformatic analyses

All GEP experiments were conducted as shown in our previous paper[28].

Microarray data performed on 30 pts were preprocessed and normalized using Robust Multi-array Average (RMA) algorithm. The Significant Analysis of Microarrays (SAM) was used to identify genes with statistically significant changes in expression in CML patients. P-values were corrected for multiple testing using false discovery rate.

GEP analysis on the BM CD34+/lin− cells of 79 CP-CML patients at diagnosis vs the same patients after 12 months of Nilotinib vs 10 healthy donors was performed using methods described in http://dx.doi.org/10.17504/protocols.io.yncfvaw.

2.7 | Statistics

2.7.1 | Sample sizing

The protocol used a single-stage phase II design, in which it is assumed that, at 6 months, the proportion of patients defining a poor response at the study drug is PP = 0.10 (eg, a poor response is assumed if only 10% or a smaller proportion of patients show complete disappearance of BM CD34+/lin−/Ph+ cells); that the proportion defining a good response is PG = 0.30 (eg, a good response is assumed if at least 30% or a greater proportion of patients show complete disappearance of BM CD34+/lin−/Ph+ cells). Under these assumptions, the study required a minimum of 41 patients. A set of 41 evaluable CCyR patients means a baseline of 69 patients (60% of which reaching CCyR, based on the ENESTnd data), and this would mean 87 patients must be enrolled since one expects that 20% of them (eg, 18, being 69 + 18 = 87) will be lost.

2.7.2 | Statistical analysis

The primary endpoint was evaluated by measuring the proportion PA of patients actually achieving the response at 6 months. The proportion was calculated together with its 95% CI. All secondary biological endpoints were evaluated by the calculus of % fractions, together with their respective 95% CIs. Analysis of Nilotinib treatment efficacy was performed as Intention to Treat (ITT) and Per Protocol (PP).

2.8 | Ethics

The PhilosoPhi34 study was conducted in accordance with the International Conference on Harmonisation Harmonised Tripartite Guidelines for Good Clinical Practice, the Declaration of Helsinki and local regulations. The protocol and informed consent forms were reviewed and approved by an independent Ethics Committee at each participating center. The PhilosoPhi34 study was registered in the EU Clinical Trials Registry (EudraCT:2012-005062-34) and ClinicalTrials.gov (NCT01856283). Eligible patients and healthy donors were included only after informed written consent was obtained.

3 | RESULTS

Eighty-seven patients were enrolled during the recruitment phase.

Patient's demographics and clinical characteristics are summarized in Table 1.

Of the 87 enrolled patients, two patients discontinued Nilotinib at <3 months of treatment while five patients discontinued the drug between 6 and 8 months. However, only 84/85 expected samples were available for analysis at 3 and 6 months; while at 12 months, all the 80 expected BM samples were available.

3.1 | Primary endpoint

At 6 months, 79 out of 84 evaluable samples showed a CCyR, of them 78 were adequate for FISH analysis on BM CD34+/lin− cells. Overall, 71 tests were evaluable for statistical analysis, reaching the number of FISH tests required (ie, a minimum of 41 evaluable

TABLE 1 Patients' characteristics and causes of discontinuation

Characteristics of 87 Pts		
Age: median (range) years	50	(18-82)
Sex: M/F	52/35	
Sokal score: Low, Intermediate, and High risk	45, 26, 16	
Time on study: mean, median (range) mo	36	32 (1-36)
Causes of discontinuation	**No. of pts**	**Time mo**
Failure	6	6, 7, 10, 12, 24, 26
Toxicity	3	1, 2, 25*
Lost to FU	6	13^, 23, 24, 26, 33, 33
Consent withdrawal	2	7, 8

Note: Out of 87 pts only six were resistant: two harbored a mutation and one was a warning.

*A lung adenoca was diagnosed. ^Patient maintained treatment and was monitored in another country. Labnet standardized laboratory was not available.

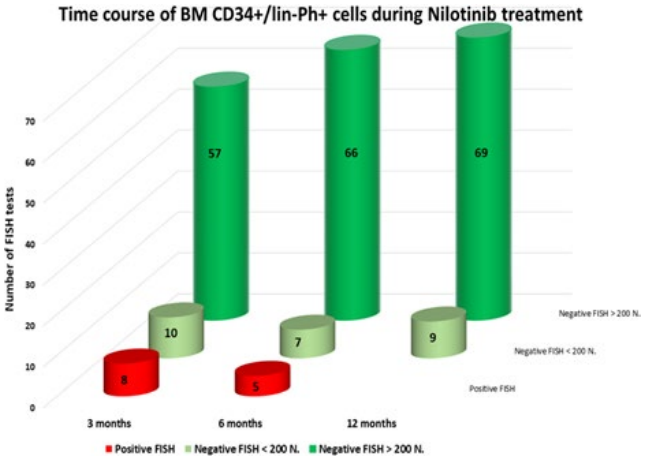

FIGURE 1 FISH analysis on CD34+/lin− selected cells at 3, 6 and 12 mo. In green negative tests with more than 200 nuclei analyzed, in light green: negative tests with less than 200 nuclei analyzed (excluded per protocol); in red: positive test. At 6 mo, we have largely exceeded the number of evaluable test required for statistical analysis (71 vs 41). Only eight FISH samples tested positive at 3 mo, only five at 6 mo, and no FISH samples tested positive at 12 mo

samples). Seven samples, that tested negative, were excluded from the statistic because less than 200 nuclei were analyzed. Only 5/71 (7%; CI 95%: 2.3-15.7%) evaluable FISH tested positive. Among these five positive patients, the Sokal score was as follows: 1 low, 3 intermediate, and 1 high.

3.2 | Biological secondary endpoints

At 3 months, 76 out of 84 evaluable samples showed CCyR and 75 were adequate for FISH analysis; at 12 months, 79 out of 80 evaluable samples showed CCyR and 78 were adequate for FISH analysis. Ten and 9 tests were excluded at 3 and 12 months, respectively, because less than 200 nuclei were analyzed. At 3 months, only 8/65 (12.3%; CI95%: 5.5%-22.8%) evaluable samples tested positive by FISH. Among them, the Sokal score was as follows: 6 low and 2 intermediate. At 12 months, none of the 69 evaluable samples tested positive by FISH (0.0%; CI95%: 0.0%-5%) (Figure 1).

At 3 and 6 months, a molecular analysis performed on patients who tested positive by FISH was ≥ 0.175% IS.

Sokal score did not predict for FISH results at any study time point. Indeed, as outlined in Table 2, a selection's bias might explain this lack of correspondence since high Sokal score patients are less prevalent among patients who achieved a CCyR, a requirement for FISH analysis.

3.3 | Evaluation of response to treatment

Seventy out of 87 enrolled patients completed the study; causes of study discontinuation were summarized in Table 1.

Six out of 87 patients failed treatment; none of them progressed to an accelerated or blastic phase. Of them, four patients failed during the first 12 months of treatment; 2 had a low Sokal (2/46 low Sokal patients, 4.3%) and 2 had a high Sokal (2/16 high Sokal patients, 12.5%). One of the low Sokal relapsed patients harbored a rare mutation, an insertion (35INS). Other two patients failed treatment: one harbored a mutation at 26 months and another one was considered as a failure at 24 months due to a persistent warning (stable MR2). The 3-month molecular ratio for the 2 low Sokal patients was 0.046% IS and 8.16% IS. Of note, the patient in MR3 response at 3 months increased the molecular ratio of 1 Log at 6 months of treatment and he is the only one of the 22 patients with MR ≥3 at 3 months who failed treatment. The 3-month molecular ratio for the 2 high Sokal patients was 11.69% IS and 47.8% IS. The fifth resistant patient, harboring an ABL mutation at 26 months of treatment, had a high Sokal; the 3-month molecular ratio was 0.5% IS. The patient in warning has a low Sokal and showed a 3-month molecular ratio of 2,723% IS.

Table 3 summarizes the ITT and PP rate of cytogenetic and molecular responses during the core phase of the study. ITT cytogenetic analysis is as follows: at 3 months CCyR 77/87 patients (88.5%); at 6 months CCyR 79/87 patients (90.8%); and at 12 months CCyR 77/87 patients (88.5%). Cytogenetic analysis was not performed in one patient at 6 and 12 months (CMR at both time points), while in a second patient, only the 12-month evaluation was missing (MR: 0.02% IS). These two patients were considered as non-responders in the ITT cytogenetic analysis and as responders in the PP analysis; moreover, considering the deep MR, the patients were considered eligible for FISH analysis on BM CD34+/lin− cells.

ITT molecular response analysis is as follows: at 3 months, MR < 10% IS 80/87 patients (91.9%); at 6 months, MR <1% IS 78/87 patients (89.65%); at 12 months, MR3 IS 67/87 (77%) and MR4.5 IS 26/87 (29.88%) patients. None of the 22 patients (including 4 H-Sokal score pts = equal proportion of study cohort) in MR3 at

TABLE 2 All CD34+/lin- FISH-positive samples: Sokal distribution.

UPN Code	Time of FISH	Cytog. Resp.	Positivity of FISH	qPCR%IS 3 mo	qPCR%IS 6 mo	qPCR%IS 12 mo	SOKAL Score
01	3 mo	**CCyR**	**1/200**	0.3000	0.0100	0.0000	**0.56**
02	3 mo	**CCyR**	**1/400**	0.3800	0.0022	0.0000	**0.86**
03	3 mo	**CCyR**	**3/300**	0.1750	0.0230	0.0050	**0.86**
04	3 mo	**CCyR**	**1/200**	0.3570	0.0333	0.0060	**0.74**
05	3 mo	**CCyR**	**2/280**	0.5710	0.0080	0.0080	**0.69**
06	3 mo	**CCyR**	**2/235**	0.2410	0.0450	0.0120	**0.57**
07	3 mo	**CCyR**	**4/150**	2.5640	0.0460	0.0177	**0.65**
08	3 mo	**CCyR**	**1/300**	8.1685	0.1224	**Fail**	**0.57**
09	3 mo	Not CCyR	29/250	3.7500	0.1900	0.0850	1.05
10	3 mo	Not CCyR	2/200	2.0600	0.2630	0.0940	1.15
11	3 mo	Not CCyR	67/70	47.8000	10.5000	**Fail**	2.40
12*	3 mo	Not CCyR	200/200	11.6900	11.2800	**Fail***	2.53
13	3 mo	Not CCyR	47/300	12.5500	0.7830	0.1370	2.18
14	6 mo	**CCyR**	**1/200**	0.2190	**0.0160**	0.0200	**0.81**
15	6 mo	**CCyR**	**1/250**	3.6900	**0.3000**	0.0900	**2.12**
16	6 mo	**CCyR**	**295/300**	0.7650	**0.0700**	0.1040	**0.78**
17	6 mo	**CCyR**	**2/200**	10.7940	**1.8138**	0.7930	**1.02**
18	6 mo	**CCyR**	**2/92**	2.7230	**0.7850**	0.9600	**0.93**
19	6 mo	Not CCyR	3/300	0.6930	1.3100	**Cons. With.**	1.36
12*	6 mo	Not CCyR	9/11	11.6900	11.2800	**Fail***	2.53
20	12 mo	Not CCyR	49/200	0.0460	0.3300	**Fail** 19.205	0.69

Note: In our analysis, we observed that Sokal score did not predict FISH analysis results on CD34+/lin cells at any time points. As per protocol, we must evaluate this test only in patients with a CCyR but this selection is, de facto, an important bias regarding Sokal score impact. If we analyzed all positive FISH samples, we observed: at 3 mo, 2/8 CCyR patients showed an intermediate Sokal but no high Sokal; 2/5 no CCyR patients showed an intermediate Sokal and three showed a high Sokal. At 6 mo, 5 CCyR patients showed 1 low Sokal, 3 intermediate Sokal, and 1 high Sokal; 2 no CCyR patients displayed a high Sokal. Globally, 1 out of 13 patients in CCyR showed high Sokal (7.69%) and five out of seven patients not in CCyR showed high Sokal (71.42%).

*only one patient tested positive at 3 and 6 mo.

We evidenced in bold the most important parameters to consider, i.e CCyR (vs noCCyR), FISH results, risk factor (Sokal score).

3 months resulted positive in the FISH analysis at 3 and 6 months and only one (low Sokal) failed treatment, harboring a 35INS.

Table 4 summarizes the MR at 24 and 36 months of treatment. In particular, at 36 months we observed these MR3.0 and MR4.5 response rates: 78.16% (ITT), 89.4% (PP), 40.22% (ITT), and 46.05% (PP), respectively.

Patients who obtained a MR3.0 at 3 months maintained a statistical higher incidence of deep molecular response over time (Figure 2).

3.4 | Gene expression profiling of BM CD34+/lin− cells and BM CD34+lin- cell counting

In this study, we aimed to perform GEP analysis on CD34+/lin− cells at diagnosis and after 12 months of Nilotinib therapy to investigate the genes and pathways that could highlight a possible correlation with the response to treatment. As a matter of fact, the majority of CD34+/lin− cells at diagnosis were Ph+ whereas the same cells after 12 months of treatment were mostly Ph−, as demonstrated by FISH analysis results.

We report here some of the results of all GEP experiments performed in the PhilosoPhi34 study.

We counted the BM CD34+/lin− cells of 30 CML patients as well as 80 CML patients at diagnosis, after 3, 6, and 12 months of Nilotinib treatment, as shown in Figure 3 and Figure 4.

Firstly, we analyzed 30 CML patients at diagnosis and after 3 and 6 months of Nilotinib. Cell counts from each patient showed some different trend of cellularity. Based on these patterns, patients were divided into two classes for GEP analysis: class 1 (n = 24) showed highly reduced levels of CD34+/lin− cells while class 2 (n = 6) demonstrated increasing levels of CD34+/lin- cells after 3 and 6 months of Nilotinib, respectively.

Bioinformatic analysis showed that when a nominal significance level alpha equal to 0.05 is adopted together with a fold-change threshold equal to 2 (absolute value), 56 transcripts were selected in the comparison between the 2 groups of CML patients.

Among them, we focused on *NFKBIA* which was overexpressed in class 1 compared with class 2.

Afterward, we performed GEP analysis of BM CD34+/lin− cells of 79 pts at diagnosis vs the same pts after 12 months of treatment vs 10 CTRLs. We demonstrated the over-expression of genes involved in the cell cycle and mitosis (n = 34), genes belonging to the JAK-STAT signaling pathway (*SOS1*, *PIK3CA*, *IL7*, *JAK2*, *STAM*, and *PTPN11*), and the ABC transporter gene *ABCD3* at diagnosis vs 12 months of Nilotinib vs CRTLs.[28]

In particular, we showed that *JAK2* was up-regulated at diagnosis vs 12 months of treatment vs CTRLs with the following *P*-values: at diagnosis vs 12 months (*P*.000036), at 12 months vs CTRLs (*P*.000285), and at diagnosis vss CTRLs (*P*.000014).

According to these data, we have considered:

- the impact of different expression of *NFKBIA* between patients at diagnosis on BM CD34+/lin- cells number during treatment,
- the correlation between *NFKBIA*, *NFKB*, and *OLFM4*, their potential impact on the stem cell survival in the niche, in particular associated with *ICAM1* activity,
- the *JAK2* role.

Consequently, we decided to verify the possible correlation between the different expression of these genes and the molecular responses over time.

In particular, we analyzed the following: the correlation between the different expression of these genes among patients at diagnosis and the molecular response over time; and the correlation between the different expression of these genes at diagnosis vs 12 months of treatment and the molecular response over time.

When we verified the impact of the different expression of *NFKBIA*, *JAK2*, *OLFM4*, and *ICAM1* at diagnosis vs 12 months of treatment and the molecular responses over time, no statistically significant differences were confirmed.

After that, we divided patients into two groups, according to *NFKBIA* and *JAK2* expression at diagnosis and after 12 months of treatment: patients who increased *JAK2* and decreased *NFKBIA* expression vs patients who decreased *JAK2* and increased *NFKBIA* expression. As we can see in Figure 5, patients of second group obtained a better MR3.0 at 3 months and a better MR 4.5 at 6 months but a statistical significance was not reached.

When we analyzed the correlation between the expression of the chosen genes at diagnosis and the molecular responses over time, we observed various impacts at different time point and different statistical correlations. In univariate analysis, Wald's test highlighted that the higher expression of *ICAM1* among patients at diagnosis correlated with optimal response (MR1.0) at 3 months (*P*.027, odds:214%) and the expression of *NFKBIA* had an inverse correlation with MR3 at 3 months (*P*.006, odds:-76%) (ie, the lower expression of *NFKBIA* correlates with the major probability of MR3.0 at 3 months). Similar analysis showed that a higher expression of *OLFM4*

TABLE 3 Cytogenetic and molecular responses intention to treat and per protocol

Response at 3 mo (ITT)	No. of pts	Rate	PP (85 pts)
MR 4.5 ≤0.0032% IS	1	1.15%	1.18%
MR 4.0 ≤0.01% IS	3	3.45%	3.52%
MR 3.0 ≤0.1% IS	22	25.28%	25.88%
MR 2.0 ≤1% IS	69	79.31%	81.17%
≤10% IS	80	91.95%	**94.11%**
CCyR	77	88.5%	**90.58%**
Response at 6 mo (ITT)	**No. of pts**	**Rate**	**PP (85 pts)**
MR 4.5 ≤0.0032% IS	15	17.24%	17.64%
MR 3.0 ≤0.1% IS	60	68.96%	70.58%
MR 2.0 ≤1% IS	78	89.65%	**91.76%**
CCyR	79*	90.80%	**94.11%**
Response at 12 mo (ITT)	**No. of pts**	**Rate**	**PP (83 pts)**
MR 4.5 ≤0.0032% IS	26	29.88%	31.32%
MR 4.0 ≤0.01% IS	41	47%	49.39%
MR 3.0 ≤0.1% IS	67	77%	**80.72%**
MR 2.0 ≤1% IS	78	89.65%	**93.97%**
CCyR	77**	88.5%	**95.13%**

*One test not done.; **Two tests not done: excluded in ITT analysis, included in PP analysis.

We evidenced in bold the percentage of patients who obtained the expected molecular response according to ELN reccomendation, at the different time points, and the corresponding rate of CCyR.

TABLE 4 Molecular responses: intention to treat and per protocol

MR	At 12 m 80 Pts	% MR		At 24 m 77 Pts	% MR		At 36 m 70 Pts	% MR	
		ITT	PP (83 Pts)		ITT	PP (81 Pts)		ITT	PP (76 Pts)
MR 4.5	26	29.88%	31.32%	34	39.08%	41.97%	35	40.22%	46.05%
MR 4.0	41	47.12%	49.39%	47	54.02%	58.02%	58	66.66%	76.31%
MR 3.0	68	78.16%	81.92%	70	80.45%	86.42%	68	78.16%	89.47%
MR 2.0	78	89.65%	93.97%	77	88.50%	95.06%	70	80.46%	92.10%

Note: Molecular response Intention to Treat and Per Protocol at 12, 24 and 36 mo. As per protocol, after the 12 mo of the core phase the treatment was at the investigator's choice. Despite 11 patients have switched to Imatinib treatment and six patients were lost to FU, deep molecular response increased over time.

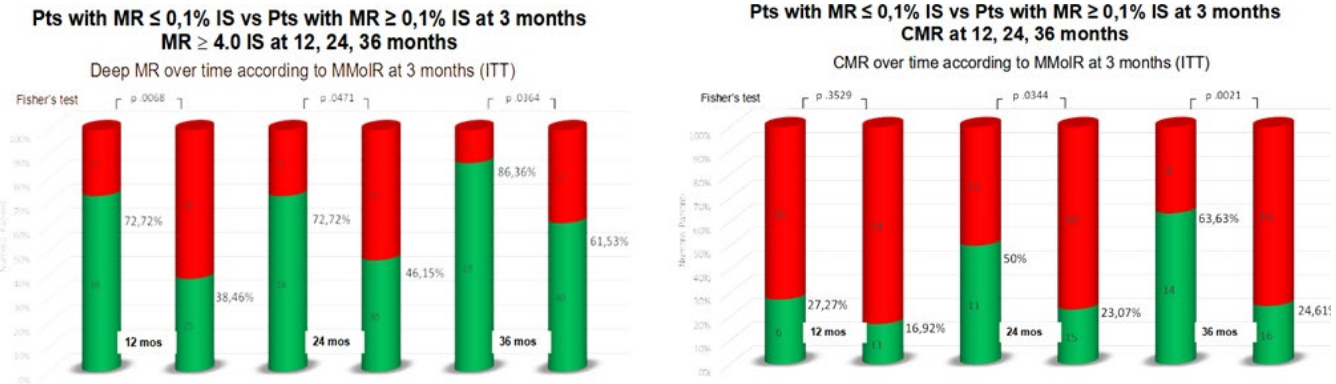

FIGURE 2 Molecular response ≥4.0 IS and Complete molecular response according to MR3.D IS. CMR: comply molecular reiponse; ITT-inrention to treat; MMolR, majormolMularreiponse. ME.4.0: out of the 87 pts, 22 pts obtained a MR3 at 3 mo of treatment. They showed a major incidence of MR≥4.0 IS at 12, 24 and 36 mo: *P*.0068, *P*.0471, *P*.0364, respectively. CMR out of the 87 pts, 22 pts obtained a MR3 at 3 mo of treatment. They showed a major incidence of CMR at 24 and 36 mo: *P*.0344, *P*.0021, respectively

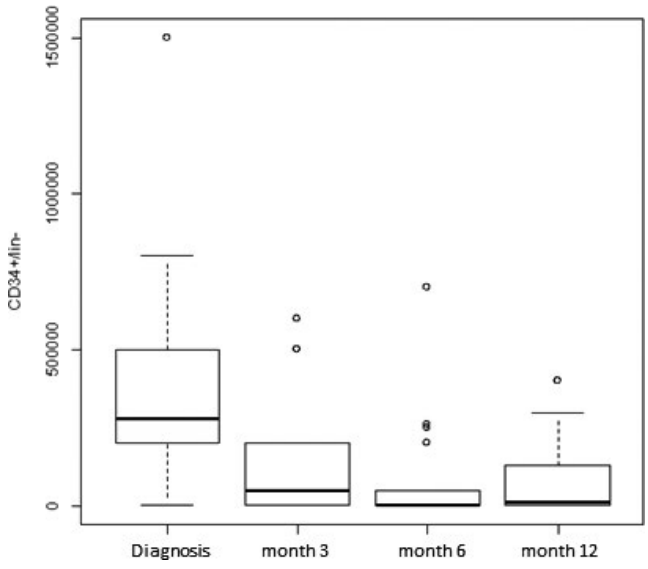

FIGURE 3 Number of BM CD34+/lin− cells of the 30 CML patients. Boxplot of the number of the BM CD34+/lin− cells of the 30 CML patients at diagnosis and after 3, 6 and 12 mo of nilotinib. The first GEP experiment was performed on these patients

correlated with MR3.0 at 12 months (*P*.023, odds:294%) (Figure 6). Multivariate analysis revealed that *JAK2*, *ICAM1*, and *OLFM4* expressions increased the statistical significance of *NFKBIA* role (*P*.002).

In order to compare our in vivo data with the in vitro published reports concerning the correlations between *NFKB* and *OLFM4*, *NFKB*, and *NFKBIA*, we performed another multivariate analysis.

The fitting test, correlating *NFKB* and *NFKBIA* expression with *OLFM4* expression at diagnosis, confirmed the up-regulation of *OLFM4* according to a lower expression of *NFKBIA* and the higher expression of *NFKB*. (Figure 7).

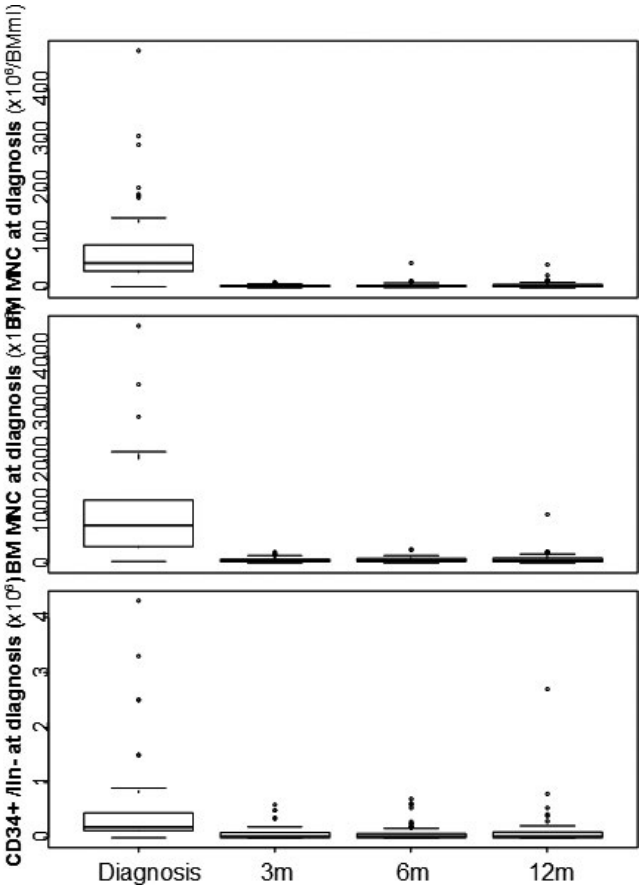

FIGURE 4 Number of BM MNCs and number of BM CD34+/lin− cells of the 80 CML patients. Boxplot of the number of BM MNCs as well as the number of the BM CD34+/lin− cells of the 80 CML patients at diagnosis and after 3, 6 and 12 mo of nilotinib. Our GEP experiments were performed on 79 out of 80 patients

4 | DISCUSSION

The aim of the PhilosoPhi34 study was to explore the in vivo impact of Nilotinib on CD34+(CD38+ and CD38-)/lin-Ph+ BM cells. In this respect, we were able to demonstrate a significant and rapid clearance of these cells from the bone marrow of treated patients. In fact, at 6 months of treatment, only 7% of evaluable FISH samples showed residual Ph+ cells (ie, 93% of the evaluable samples tested negative). These data markedly differed from published data on Ph+

leukemic cells clearance in patients in sustained CCyR and MMR, during Imatinib treatment [12], who tested positive by FISH in approximately 45% of cases at 39 months (median of treatment). Although the PhilosoPhi34 study is not a comparative study, our data support the concept that Nilotinib seems more effective than Imatinib in clearing Ph+ leukemic cells (Table 5).

In our statistic design, a good response was assumed if complete clearance of BM CD34+/lin-/Ph+ cells by FISH could be demonstrated after 6 months of treatment in at least 30% of patients in CCyR and in a minimum of 41 evaluable samples. Our results showed that 93% of the 71 evaluable samples tested negative at 6 months of treatment.

Furthermore, Nilotinib treatment results in an early and stable clearance of CD34+/lin-Ph+ BM cells with only 12.3% of evaluable FISH tested positive at 3 months and none of the 69 evaluable FISH tested positive at 12 months.

These results are consistent with clinical data. In fact, at 3 months, about 90% of patients obtained a CCyR and more than 25% obtained a MMR IS; at 12 months, about 95 % of patients showed a CCyR and 49% of patients showed a MR4 IS. Patients who obtained a MMR at 3 months maintained a higher probability of deep molecular response at 24 (72.72%) and 36 months (83.36%).

Overall, our study confirmed the efficacy and safety profile of Nilotinib as a first-line treatment of CP-CML: During the "core" phase (12 months of treatment), only 8/87 enrolled patients (9.2%) discontinued the drug: toxicity (two patients), resistance (four patients), and consent withdrawal (two patients). In addition, results of ITT and PP analysis did not significantly differ at any time point. Of relevance, at 12 months, comparative analysis of ITT and PP results shows CCyR 88.5% (two test not performed in two CMR patients) vs 95,13%; MMR IS 77% vs 80.72%; MR4.5 IS 29.88% vs 31.32%. Of note, at a median time on study of 32 months (mean, 36 months) none of the patients had progressed to accelerated or blastic phase, confirming that a rapid disease burden debulking (ie, earlier and deeper response) protects patients from disease progression, including those with a high Sokal score.

While literature data are unanimous in confirming the positive impact of Nilotinib on CML proliferative cells in terms of both efficacy and mechanism of action, conflicting results on the in vitro and in vivo impact on leukemic progenitor and stem cells (CD34+CD38+ and CD34+CD38−) still exist.

It is difficult to trace back the significant in vivo impact of Nilotinib on CD34+ (CD38+ and CD38−)/lin-Ph+ cells to its recognized mechanism of action.

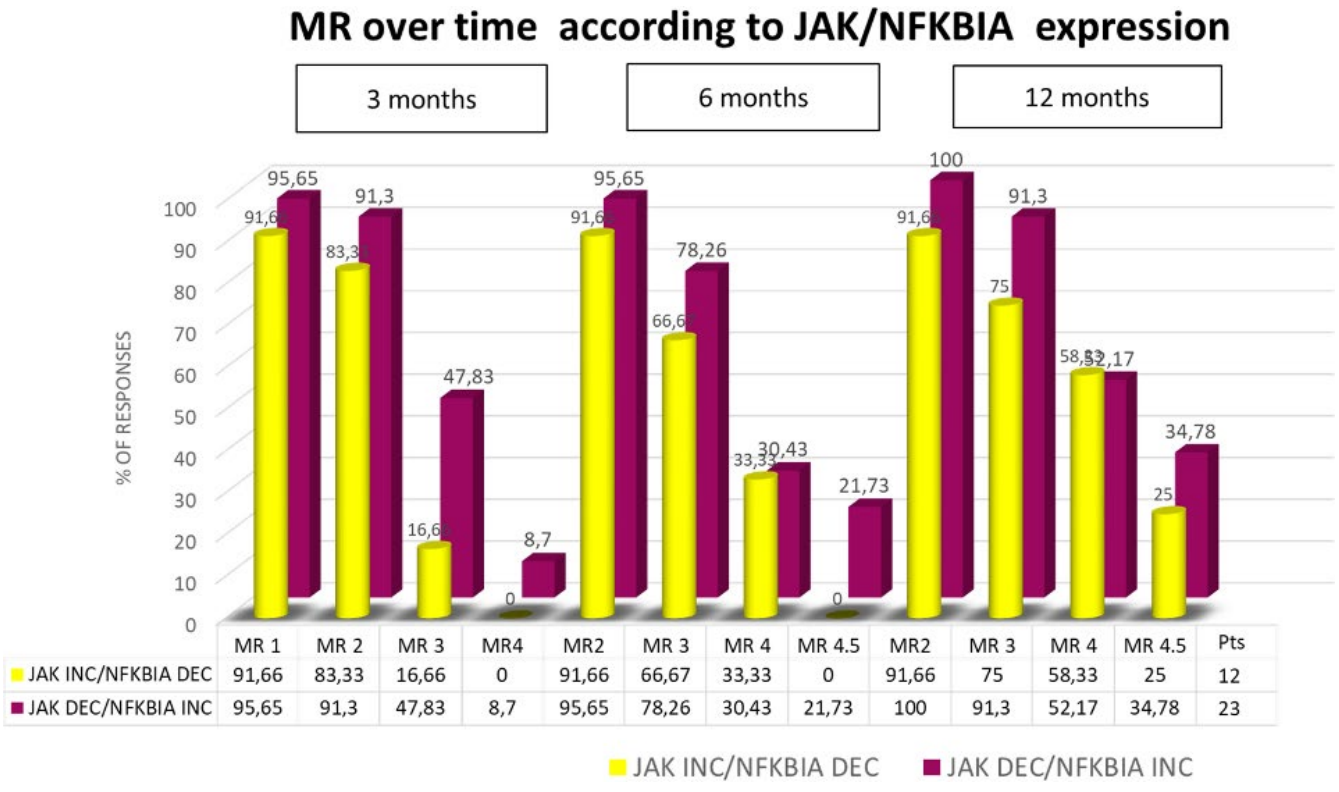

FIGURE 5 Molecular response during the core phase according to the*JAK2* and *NFKBIA* expressions. Here, we compared two groups of patients selected according to the significant different expressions of genes at diagnosis and after 12 mo of nilotinib treatment. Patients in group 1: both increased *JAK2* expression and decreased *NFKBIA* expression (12 pts); patients in group 2: both decreased *JAK2* expression and increased *NFKBIA* expression (23 pts). The molecular response over time clearly differed between the two groups at 3 and 6 mo of treatment: MR3.0 at 3 mo and MR4.5 at 6 mo showed 47.83% vs 16.66% and 21.73% vs 0%, respectively. Despite this evidence, statistical significance was not reached probably due to a few numbers of cases in the groups

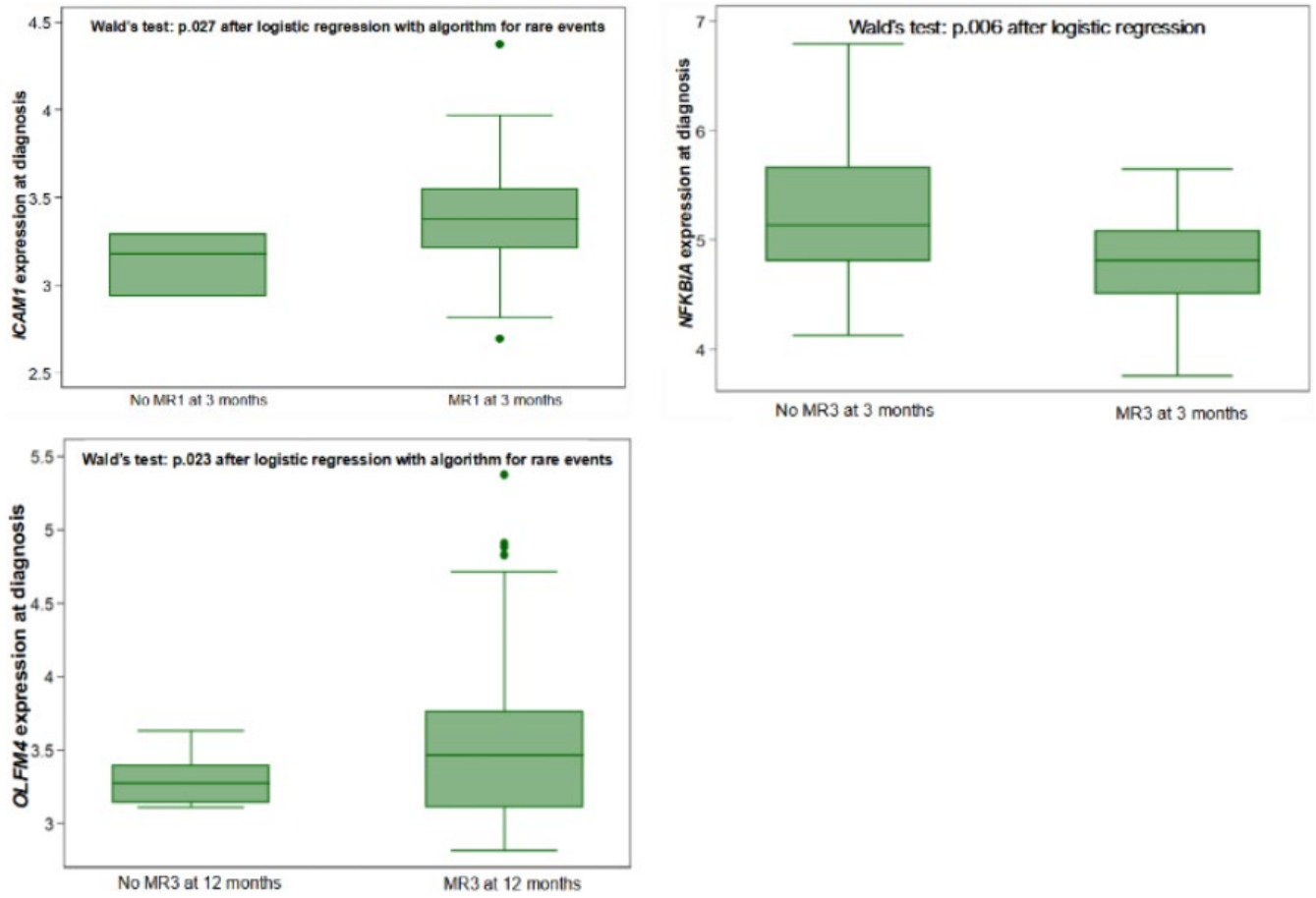

FIGURE 6 Correlation between different genes expression and molecular response. In this univariate analysis, we observed that the different expression of these three genes at diagnosis correlated with a better molecular response overtime. In particular, at 3 mo, each unitary increase in *[CAM]* corresponds to a 214% increase in the odds of the event (*P*.027); at 12 mo, each unitary increase in *OLFM4* corresponds to a 294% increase in the odds of the event (*P*.023); at 3 mo, each unitary increase in *NFKBIA* corresponds to a 76% decrease in the odds of the event (*P*.006). This indicate that only a lower *NFKBIA* expression correlate whit a better MR3 at 3 mo. In multivariate analysis, this significance statistically increased if we consider both *NFKBIA* and *JAK2* expressions (*P*.004). If we consider *NFKBIA*, *JAK2*, *[CAM]* and *OLFM4* expressions, together. The probability of MR3 at 3 mo further raise (*P*.002)

Nilotinib targets the BCR/ABL protein which is known to be expressed at a low level in CD34+/lin− CML cells. Moreover, in vitro data suggest that Ph+ stem cells have a mechanism of survival which is independent of BCR/ABL [19,20,29]. Therefore, alternative or complementary actions of Nilotinib on Ph+ stem cells, such as an apoptotic effect, an exhaustion of the CML stem cells, or an impact on the homeostasis of the hematopoietic niche, could be hypothesized.

These alternative mechanisms of action may be mediated by either off-target effects of Nilotinib, or by the different impact of Nilotinib on CD34+/lin− cells gene expression linked to a different predisposition of the leukemic and/or physiological stem cells.

In order to investigate these hypotheses, a gene expression profiling study complemented the PhilosoPhi34 study. GEP represents a unique tool for the analysis of changes in the transcriptional profile of leukemic cells highlighting a specific biological behavior, a stage- and disease-specific signature, or responses to therapy[30]. GEP studies have been proven helpful to classify disease subtypes and to predict treatment response and outcomes in leukemias and lymphomas[31,32]. Several studies on CML gene expression profiling have been performed in order to determine: (a) stage-specific patterns[33-35], (b) specific gene set in CML CD34+ cells [36-39], and (c) Imatinib response prediction [40,41]. Although multicenter studies have successfully identified a robust predictive gene set[42], no common gene sets have been found yet.

In our study, GEP analyses highlighted a different expression of *NFKBIA* among patients at diagnosis and a correlation between its expression and the decrease of the number of CD34+/lin− cells during treatment.

These results suggest a possible role of *NFKBIA* in influencing leukemic stem cell survival and a subsequent response to treatment. Indeed, *NFKBIA* is involved in apoptosis (*PI3K and NFKB*) and encodes the IkBα protein, which is an inhibitor of *NFKB* and plays a well-recognized role in regulating normal cell survival and proliferation.

A previous in vitro study[43] demonstrated that the blockade of *NFKB* increased the sensitivity to TKIs of the BCR-ABL transformed

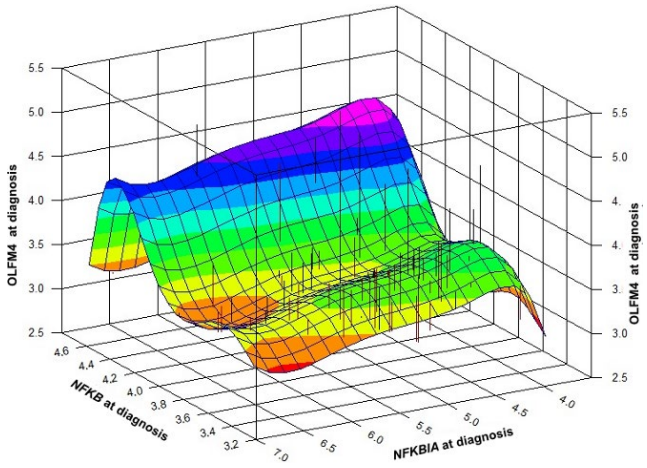

FIGURE 7 *OLFM4* expression according to *NFKB* and *NFKBIA* expression, at diagnosis. This 3D scatter plot showed an analysis of *OLFM4* as a function of both *NFKB* and *NFKBLA* at diagnosis. As reported only in vitro till now. *OLFM4* amplified as a function of *NFKB* and also decrease at increasein value of *NFKBIA*. This second condition is less evident according with other in vitro data that indicate *NFKBIA* as an indirect regulatory agent of *OLFM4* which is regulated by *NFKB*, that in turn, is down regulated by *NFKBIA*

TABLE 5 Probability of BM CD34+/lin- cells Ph negativity by FISH

Ph- FISH on BM CD34+/lin- cells (PhilosoPhi34 vs Bocchia 2008)		
	Nilotinib	**Imatinib**
Status	CCyR at 6 mo	CCyR at 39 mo (24-59)
FISH Ph- pts/Tot. pts	66/71 (93%)	17/31 (55%)
IC 95%	87.3%-97.6%	36%-72.6%
IC 99.9%	75.5%-99%	25.6%-81.8%

Note: We reviewed data reported by Bocchia et al concerning the CD34+/lin-Ph+ clearance during at least 24 mo of Imatinib treatment. In this study, the best probability of FISH negativity was 72.6%. In our study, after 6 mo of Nilotinib treatment, the worst probability of FISH negativity (87.3%) exceeds that data. This strongly suggests the superiority of Nilotinib vs Imatinib in clearing Ph+ stem cells.

lymphoblasts. Moreover, *OLFM4* which is a candidate survival factor for CML primitive cells[29] requires interaction with *NFKB*[44].

Herein, the observed higher expression of *NFKBIA* in the CD34+/lin− cells at diagnosis seems to be associated with an initially higher sensitivity to treatment, as demonstrated by the decrease of the number of the CD34+/lin− cells. In terms of clinical response to treatment, the decreased level of *NFKBIA* expression observed between diagnosis and 12 months of treatment seems to be associated with a faster and deeper molecular response but the statistical significance was not reached. Moreover, the lower expression of *NFKBIA* at diagnosis statistically correlated with the higher probability of MR3.0 at 3 months. These apparently conflicting data might be explained according to the *NFKBIA* role.

NFKBIA is involved in 68 pathways regulating apoptosis (*PI3K and NFKB*) and encodes IkBα protein down-regulating *NFKB* which is a potential downstream target of BCR-ABL due to its positive role in cell survival and proliferation. Consequently, we must suppose that the higher expression of *NFKBIA* in CD34+/lin−Ph+ cells affects proliferation even after a rapid debulking due to TKI treatment. This hypothesis was confirmed by our data on 30 patients at diagnosis that showed a correlation between *NFKBIA* over-expression and highly reduced levels of CD34+/lin− cells in BM. This cell reduction is not necessarily correlated with a better response. In fact, the decrease expression of *NFKBIA* after 12 months of treatment has not a correlation with response. Considering that the majority of CD34+/lin− cells at diagnosis were Ph+ whereas the same cells after 12 months of treatment were mostly Ph−, we suggest that CD34+/lin−Ph+ cells over-expressing *NFKBIA* are not more sensitive to treatment. By contrast, the up-regulation of *NFKBIA* after 12 months of treatment indicates that the CD34+/lin−Ph+ cells down-regulate this gene compared with the normal cell counterpart. This condition showed a better trend of response but not yet a significant statistic correlation. Conversely, the lowest expression of *NFKBIA* in CD34+/lin−Ph+ cells statistically correlated with a deep and faster response. A better correlation was observed if *JAK2*, *OLFM4*, and *ICAM1* were up-regulated. *JAK2* sustains proliferation while *OLFM4* and *ICAM1* promote stem cells anchoring to the nice.

For several years, we have been presuming the existence of different biological CML subgroups with different behavior according to the outcome of patients.

Herein, we have confirmed in vivo, on 79 patients, that the highest and lowest expression of *NFKB* and *NFKBIA*, respectively, matches with the highest expression of *OLFM4*. Contrary to the expected, we have observed better responses when these genes were expressed at the level that promote both proliferation and stem cells anchoring. In other terms, when the Ph+ cells more sensitive to TKI expand (proliferating cells), those less sensitive were anchored (stem cells pool).

Focusing on the reported data and considering the above speculation, the choice of a more specific and potent TKI at diagnosis, such as Nilotinib, is crucial to increase deep and faster molecular response and the probability of discontinuation and TFR. Moreover, these data could be a background for a new different therapeutic strategy for CML treatment.

According to our evidences, different scenarios can be considered.

In fact, we can suppose a combined treatment aimed “to reproduce” the biological conditions of the better responder sub-group during treatment (a) and at diagnosis (b) for the following patients:

a. those patients who have not obtained a deep and stable molecular response (issue: TFR) or were not in optimal response;
b. those patients who have not the better biological assessment (if tested) or for those patients who need to obtain an early TFR in order to reduce the exposure to TKI (comorbidities).

c. Phase II studies in resistant patients are needed in order to identify the best combined treatment for these goals.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Contribution: E.P. designed, performed and coordinated the research, collected, contributed, analyzed, and interpreted the data, and wrote the paper; M.N. performed the statistical design and performed statistical analyses; G.D.C performed FISH analysis and interpreted data; A.T. designed the exploratory study, performed GEP analysis, and wrote the paper; B.D.C. performed the bio statistical analyses (GEP data); R.C. analyzed and interpreted data, analyzed the results, and critically revised the paper. All authors collected data and revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. Microarray data supporting this study are available in the GEO (gene expression omnibus).

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