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Director: Prof. Alessandra Renieri

Evaluation of residual CD34+Ph+ progenitor cells in chronic myeloid leukemia patients in complete cytogenetic response during first line nilotinib therapy

Tutor

Prof. Monica Bocchia

Dissertation of

Dr. Marzia Defina

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INTRODUCTION

Chronic myeloid leukemia (CML) is an uncommon malignancy, the treatment and prognosis of which have dramatically shifted over the last decade. Characterized by a translocation between chromosomes 9 and 22, known as the Philadelphia chromosome, small-molecule tyrosine kinase inhibitors (TKIs) targeted against the oncogenic BCR-ABL fusion protein have changed this once fatal disease into a potential "curative" disease and into the model of targeted therapy.

The first generation TKI, imatinib mesylate is, in fact, highly effective in reducing leukemic cell burden inducing rapid hematologic and cytogenetic responses in the vast majority of patients. While its efficacy it has been widely confirmed, it has been also demonstrated that discontinuation of treatment is associated with molecular relapse in about 60% of patients even if they had previously achieved a sustained complete molecular response (CMolR)[1]. The cause of CML reappearance could reside in the persistence of TKIs resistant leukemic stem cells, representing a "quiescent" reservoir of the disease. On this regard it has been reported that BCR-ABL positive progenitor cells (identified as $CD34^+Ph^+$ cells) can still be detected in patients in complete cytogenetic response (CCyR) not only after short term of imatinib treatment[2] but also after a stable long lasting CCyR[3]. In fact, we had previously performed a study in which we evaluated the presence of residual bone marrow $CD34^+Ph^+$ cells in 31 CML patients in CCyR for a median time of 35 months during imatinib treatment. The study demonstrated that 45% of patients (the majority of which were also in Major Molecular Response –MMolR) still harboured a median of 1% (range 1-7) of $CD34^+Ph^+$ CML cells in the bone marrow[3]. Nilotinib, a second generation TKI, has a greater potency and selectivity for BCR-ABL than imatinib[4] and was first approved for patients with CML in chronic or accelerated phase who were resistant to or could not tolerate imatinib[5]. Furthermore a recent prospective study comparing nilotinib with imatinib as first line treatment in CML patients has confirmed considerable efficacy both in terms of CCyR and major molecular response (MMolR)[6]. Nilotinib appears to eradicate more rapidly the bulk of CML cells, at both dosages of 300 mg BID and 400 mg BID, inducing in early chronic phase patients a higher rate of CCyR at 6 months of treatment compared to imatinib (67% and 63% vs 45% respectively)[7]. This superior efficacy has been so far confirmed also at 24 months of treatment with a CCyR rate of 87% and 85% vs 77%

and a MMolR rate of 71% and 67% vs 44% respectively[8]. Despite these very promising results, up to date it is unknown if nilotinib would be more effective in eradicating CML $CD34^+$ cells hence representing a potential "curative" treatment for CML patients. On this matter *in vitro* data showed that nilotinib, as well as imatinib, is unable to eliminate CML progenitors[9,10]. However no reports have been published so far about the evaluation of persisting Ph^+ progenitor cells in CML patients during first line nilotinib treatment.

AIM OF THE STUDY

In order to compare the efficacy of nilotinib in eliminating CML precursors with respect to imatinib, we investigated whether bone marrow $CD34^+Ph^+$ progenitors could still be detected in a cohort of CML patients in CCyR following front line nilotinib therapy. Others additional objectives of this study are:

- correlation of the percentage of residual $CD34^+Ph^+$ cells with molecular response;
- validation of FISH analysis of bone marrow isolated $CD34^+$ cells as alternative method for the study of minimal residual disease in CML.
- characterization of the "stemness state" of $CD34^+$ cells, evaluating CD38 expression that is a marker involved in many cellular process like adhesion and intra-cellular signaling[11] and that characterizes a more differentiate cell.

MATERIALS AND METHODS

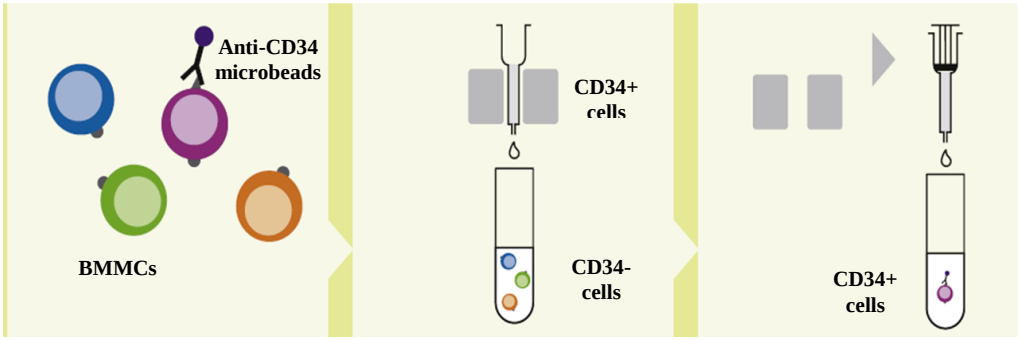
Patients' population

Endpoint of the study was to evaluate the percentage of bone marrow residual $CD34^+Ph^+$ cells in CML patients in CCyR during first line nilotinib treatment. The patients studied were included in 2 clinical trials (GIMEMA CML0307 study, ClinicalTrials.gov number NCT00481052 and CAMN107A2303 study, ClinicalTrials.gov number NCT00471497) and the evaluation of residual leukemic stem cells was performed during a routine bone marrow aspirate after receiving a specific patient informed consent. As in our previous $CD34^+Ph^+$ study during imatinib treatment[3] about 10mls of bone marrow (BM) was collected in heparin anticoagulated tubes per each patient. A small amount of the sample was not further manipulated and was evaluated for standard FISH analysis and flow cytometry study. The rest of the sample was used for $CD34^+$ isolation and subsequent FISH analysis of $CD34^+$ purified cells.

Magnetic labeling and separation of $CD34^+$ progenitors

Bone marrow mononuclear cells (BMMCs) were isolated by density gradient separation and $CD34^+$ cells were selected from BMMCs using immunomagnetic column separation according to published methods and manufacturer instructions (Miltenyi Biotech, Auburn, CA) and as previously published[3]. Briefly, BMMCs were resuspended in buffer (PBS; 0,5% FCS; 2mM EDTA) to obtain a concentration of 10^8 total cells/300μl. Subsequently 100μl CD34 microbeads per 10^8 total cells and 100μl/10^8 total cells of Fc receptor blocker were added to the cell suspension. After incubation for 30 min at 4°C, cell suspension was washed with buffer and applied to the immunomagnetic column at a concentration of approximately 10^8 total cells/500μl. In order to achieve a highly purified $CD34^+$ population two rounds of magnetic separation were performed. [Fig. 1]

Fig. 1



Magnetic labelling with anti-CD34 microbeads

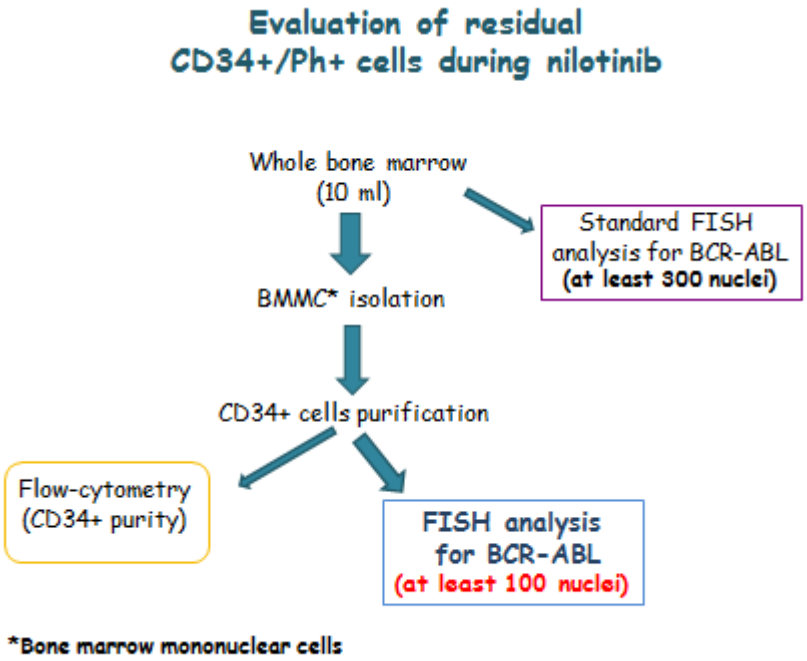
Magnetic column separation (CD34+ cells "stick" to the column)

Eluition of labelled cells

Flow Cytometry analysis of CD34$^+$ cells

To determine the yield and the purity of sorted CD34$^+$ cells, aliquots of whole bone marrow were evaluated for CD34$^+$ cells by flow cytometry study. Similarly, aliquots of cells were analyzed after column separation [Fig 2]. Flow cytometry analysis was performed by incubating each cell sample with an anti-CD34, anti-CD38 and anti-CD45 fluorescent antibodies and subsequently by analyzing the samples on a FACScan flow cytometer (BD Biosciences, San Josè CA USA).

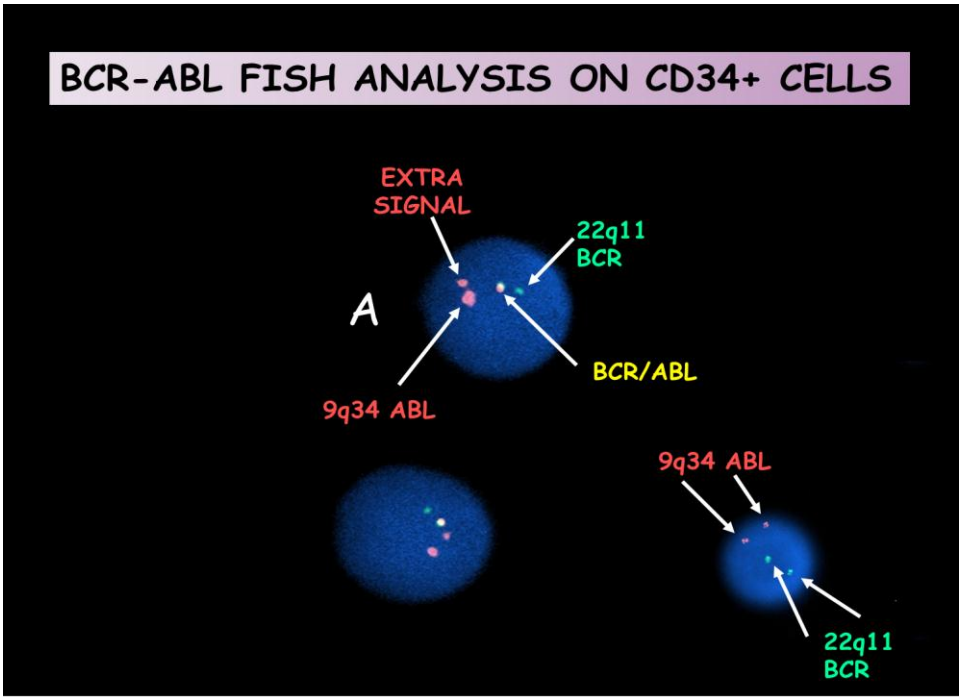
Fig. 2



FISH analysis of whole bone marrow cells and purified CD34^{+} cells

FISH was performed on fixed cells according to conventional published methods and manufacturer's specifications. Briefly, slides were denatured in 70% formamide/2x SSC for 3 minutes at 75°C and deydrated in ethanol solutions. About 10µl of probes were hybridized on interphase cells overnight at 42°C and washes performed in 2 x SSC and Tween/2x SSC, counterstained with 4'-6'-diamidine-2-phenylindole (DAPI). LSI BCR/ABL Dual color extra signal (ES), single fusion translocation was used as probe (Vysis, Downers Grove,IL, USA). Slides were analyzed with a Nikon 2 fluorescence microscope and images captured with a CCD camera using image analysis system (Genikon). When conventional whole bone marrow analysis was performed, at least 200 interphase cells were analyzed. In case of CD34^{+} purified cells 100 interphase nuclei was considered an adequate number for FISH analysis. Two observers independently scored only isolated cells in order to avoid possible false positive results (overlapping nuclei). As in our experience the LSI BCR-ABL ES probe has a false positive signal rate close to 0, FISH negativity was defined as the complete absence of BCR-ABL fusion signal. [Fig.3]

Fig. 3



RESULTS

The results of our study have been published in *Cancer* on April 2012 and the paper is reported:

Original Article

Evaluation of Residual $CD34^+Ph^+$ Progenitor Cells in Chronic Myeloid Leukemia Patients Who Have Complete Cytogenetic Response During First-Line Nilotinib Therapy

Marzia Defina, MD[1]; Micaela Ippoliti, BS[1]; Alessandro Gozzetti, MD[1]; Elisabetta Abruzzese, MD[2]; Fausto Castagnetti, MD[3]; Rosaria Crupi, BS[1]; Mario Tiribelli, MD[4]; Massimo Breccia, MD[5]; Marzia Salvucci, MD[6]; Lara Aprile, MD[1]; Claudia Baratè, MD[7]; Antonella Gozzini, MD[8]; Gianantonio Rosti, MD[3]; Francesco Lauria, MD[1]; and Monica Bocchia, MD[1]

BACKGROUND: Compared with imatinib, nilotinib is a potent breakpoint cluster region/v-abl Abelson murine leukemia viral oncogene (bcr-abl) kinase inhibitor, and it induces higher rate and rapid complete cytogenetic response (CCyR), yet no clinical data are available regarding its efficacy against chronic myeloid leukemia (CML) stem cells. Earlier studies demonstrated that clusters of differentiation 34–positive, Philadelphia chromosome–positive ($CD34^+Ph^+$) cells are detectable in about 45% of patients with CML, despite being on long-term imatinib therapy and having achieved sustained CCyR. **METHODS:** $CD34^+$ cells from bone marrow of de novo CML patients in the chronic phase (n = 24) treated with nilotinib (median duration of therapy, 22 months) were isolated and scored for BCR-ABL by fluorescent in situ hybridization (FISH) analysis. Similar analysis was also performed in 5 de novo CML chronic phase patients who achieved CCyR within 3 months of nilotinib therapy. **RESULTS:** FISH evaluation of a median of 100 $CD34^+$ nuclei per patient revealed that only 1 of 20 (5%) evaluable patients showed residual Ph^+ progenitor cells. In this patient, just 1 of 140 (0.7%) $CD34^+$ interphase nuclei was found to be positive for BCR-ABL. Surprisingly, no $CD34^+Ph^+$ cells were found even in those 5 patients evaluated after 3 months of nilotinib treatment. **CONCLUSIONS:** This study assessed for the first time the persistence of $CD34^+Ph^+$ cells during nilotinib first-line treatment. Preliminary results showed that in patients in CCyR, even after short-term nilotinib therapy, residual leukemic progenitors are very rarely detected compared with imatinib-treated CCyR patients. It is yet to be determined if these findings will have an impact in the path to a cure of CML with tyrosine kinase inhibitors. *Cancer* 2012;118:5265-9. © *2012 American Cancer Society.*

KEYWORDS: chronic myeloid leukemia, nilotinib, leukemic stem cells, $CD34^+$ cells.

Imatinib mesylate is highly effective in reducing leukemic cell burden in chronic myeloid leukemia (CML), inducing rapid hematologic and cytogenetic responses in the vast majority of patients. Although its efficacy has been widely confirmed, it has also been demonstrated that discontinuation of treatment is associated with molecular relapse in about 60% of patients, even if they had previously achieved a sustained complete molecular response (CMolR).[1] The cause of CML reappearance could reside in the persistence of tyrosine kinase inhibitor (TKI)-resistant leukemic stem cells, representing a "quiescent" reservoir of the disease. In this regard, it has been reported that BCR-ABL–positive progenitor cells (identified as clusters of differentiation 34–positive, Philadelphia chromosome–positive [$CD34^+Ph^+$] cells) can still be detected in patients with complete cytogenetic response (CCyR) not only after short-term imatinib treatment[2] but also after a stable, long-lasting CCyR.[3] In fact, we had previously performed a study in which we evaluated the presence of residual bone marrow (BM) $CD34^+Ph^+$ cells in 31 CML patients in CCyR for a median time of 35 months during imatinib treatment. The study demonstrated that 45% of patients, the majority of whom were also in major molecular response (MMolR), still harbored a median of 1% (range, 1%-7%) of $CD34^+Ph^+$ CML cells in BM.[3] Nilotinib, a second-generation TKI, has a greater potency and selectivity for BCR-ABL than does imatinib[4] and was first approved for patients with CML in the chronic or accelerated phase who were resistant to or could not tolerate imatinib.[5] Furthermore, a recent prospective study comparing nilotinib with imatinib as first-line treatment in patients with CML has confirmed considerable efficacy

Corresponding author: Marzia Defina, MD, Department of Hematology, University of Siena, Policlinico "Le Scotte," viale bracci 16, 53100 Siena, Italy; Fax (011) 39 0577586185; marziadefina@libero.it

[1]Department of Hematology, University of Siena, Siena, Italy; [2]Department of Hematology, S. Eugenio Hospital, Tor Vergata University, Rome, Italy; [3]Department of Hematology/Oncology "L. and A. Seragnoli," University of Bologna, Bologna, Italy; [4]Hematology and Transplants Center, Udine, Italy; [5]Department of Cellular Biotechnologies and Hematology, University of Rome "La Sapienza," Rome, Italy; [6]Santa Maria delle Croci Hospital, Ravenna, Italy; [7]Department of Hematology, University of Pisa, Pisa, Italy; [8]Department of Hematology, Azienda Ospedaliero Universitaria Careggi, University of Florence, Florence, Italy

We thank all additional authors who participated in the study: Michela Rondoni, Department of Hematology, University of Siena, Siena, Italy; Michele Baccarani, Department of Hematology/Oncology "L. and A. Seragnoli," University of Bologna, Bologna, Italy; Alfonso Zaccaria, Santa Maria delle Croci Hospital, Ravenna, Italy; Renato Fanin, Hematology and Transplants Center, Udine, Italy; Valeria Santini, Hematology, AOU Careggi, University of Florence, Florence, Italy; Malgorzata Monika Trawinska, Department of Hematology, S. Eugenio Hospital, Tor Vergata University, Rome, Italy; and Giuliana Alimena, Department of Cellular Biotechnologies and Hematology, University of Rome "La Sapienza," Rome, Italy. We thank Kathryn Smith for language revision.

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Patients' population

Twenty-four patients were evaluated for residual $CD34^{+}Ph^{+}$ leukemic cells and their disease and treatment schedule are summarized in Table 1.

Tab. 1

Tot. Patients		24
Median age		47 (29-80)
M/F		18/6
Median time of nilotinib treatment (months)		22 (9-30)
Treatment schedule	400mg BID	17/24 (71%)
	300mg BID	5/24 (21%)
	400mg/die (intolerance)	2/24 (8%)
Median CCyR duration (months)		17.5 (6-27)
MMolR at time of analysis		20/24 (83%)
Median MMolR duration (months)		12 (1-27)

The median age at CML diagnosis was 47 years (range 29-80) with 18 males and 6 females. At the time of residual $CD34^{+}Ph^{+}$ evaluation, the median time of nilotinb treatment was 22 months (range 9-30) with 17 patients (71%) receiving nilotinib 400mg BID, 5 (21%) 300mg BID while 2 patients (8%) were on 400mg daily due to intolerance. All patients had been in CCyR for a median time of 17.5 months (range 6-27); 20/24 patients (83%) had been in MMolR (i.e. BCR-ABL/ABL^{IS} ratio <0.1%)[11, 12] for a median time of 12 months (range 1-27); 1/24 (4%) had been in CMolR (i.e BCR-ABL/ABL^{IS} ratio <0.01%)[12,13] for 12 months, while 3/2 had not yet achieved a MMolR.

A detailed description of each patient characteristics and response to treatment is included in Table 2.

Tab. 2 Patients' characteristics, disease response to nilotinib and FISH analysis results at the time of $CD34^{+}Ph^{+}$ cell evaluation

Pts	Sex/Age	Months on nilotinib	Nilotinib dosage	Months of CCyR	MMolR	Months of MMolR	FISH CD34+ N° nuclei Ph+/ N° nuclei analyzed
1	M/42	15	800	8	No	0	0/100
2	F/35	16	600	13	Yes	11	0/60
3	M/46	16	600	13	Yes	10	0/80
4	M/58	19	800	14	Yes	10	N.E.
5	M/40	21	800	18	No	0	0/300
6	M/56	28	800	25	Yes	22	0/100
7	M/48	21	800	14	Yes	1	0/75
8	M/77	20	800	17	Yes	14	0/200
9	F/26	20	800	16	Yes	13	0/300
10	M/55	22	800	17	Yes	14	0/100
11	M/44	28	800	25	Yes	10	0/100
12	M/40	24	800	21	Yes	21	1/140
13	M/73	27	600	22	Yes	22	N.E.
14	F/58	22	800	15	CMolR	12	0/100
15	M/80	18	600	15	Yes	15	0/100
16	M/64	9	600	6	Yes	3	0/100
17	M/50	20	800	17	Yes	12	0/300
18	F/29	29	400	26	Yes	3	N.E.
19	F/42	30	800	27	Yes	27	0/65
20	M/50	30	800	27	Yes	21	0/100
21	F/44	29	800	26	Yes	26	0/86
22	M/60	30	800	27	Yes	12	0/100
23	M/47	30	400	27	Yes	18	0/100
24	M/47	30	800	27	No	0	N.E.

CCyR: complete cytogenetic response; MMolR: major molecular response; CMolR: complete molecular response; FISH: fluorence in situ hybridization; N.E.: not evaluable

Yield and immunophenotypic analysis of $CD34^+$ population

Twenty-four BM samples were collected for $CD34^+Ph^+$ cells evaluation harvesting a median volume of 10 mls (range 8-14). Total median cellularity was $20x10^6$/ml (range $5x10^6$- $42x10^6$) and the median percentage of $CD34^+$ as measured by flow-cytometry was 0.64% (range 0,03-1,36%) of total bone marrow cells. After immunomagnetic column separation a median number of $4,5x10^5$ (range $0,9x10^5$-$1,5x10^6$) of $CD34^+$ cells was collected thus resulting in a yield of about 50% of the expected number. Purity of $CD34^+$ enriched population resulted in a median value of 89% (range 43-89) [Fig. 4]. Regarding CD38 antigen expression, a median of 86% (range 23-97) were $CD34^+CD38^{high}$ cells while a median of 14% were $CD34^+CD38^{low}$ [Fig 5].

Fig. 4

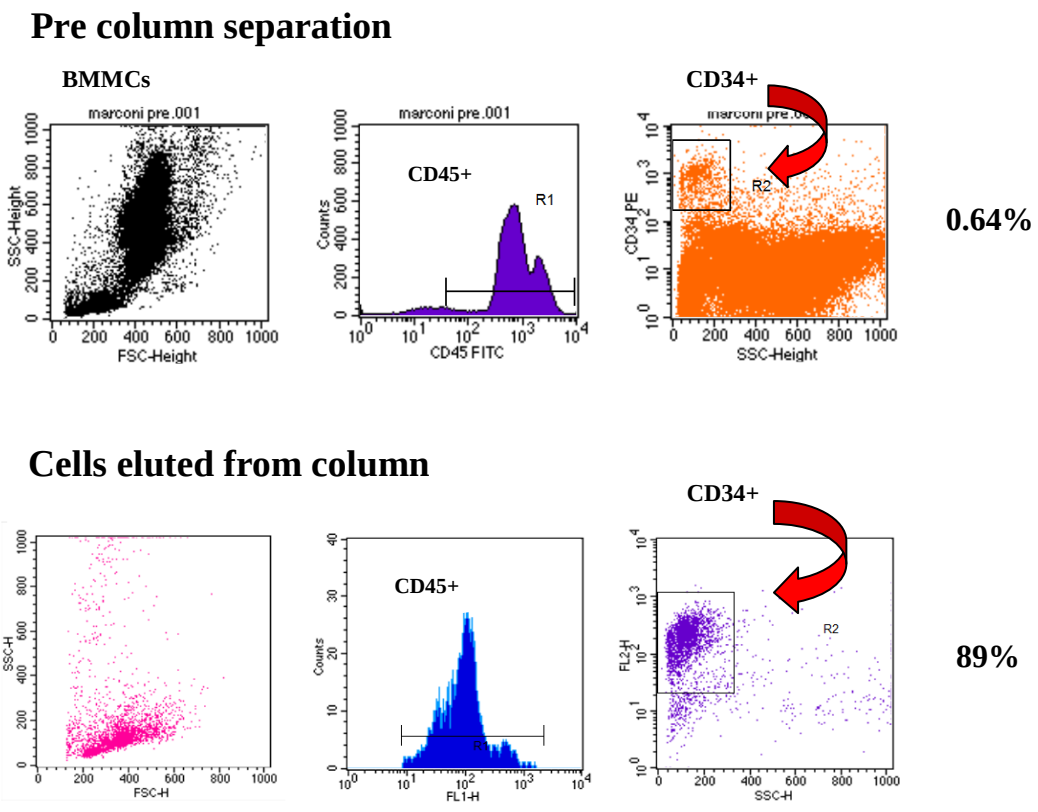
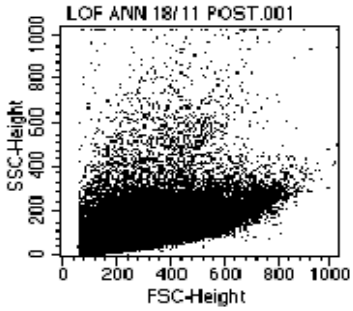
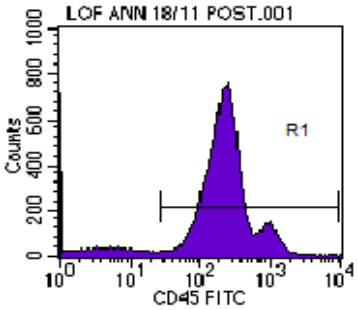
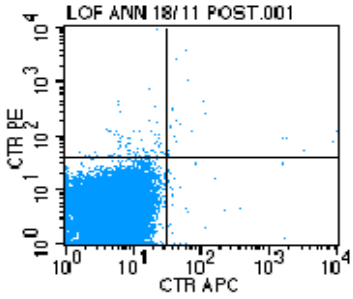
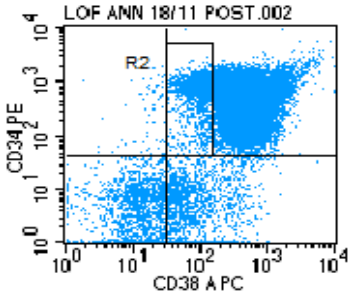


Fig. 5









Quac

File: LOF ANN 18/11 POST.001
Sample ID:
Tube:
Acquisition Date: 19-Nov-09
Gated Events: 98624
X Parameter: FL4-H CTR APC (Log
Quad Location: 29, 40

Quad	Events	% Gated	% Tota
UL	265	0.27	0.25
UR	106	0.11	0.10
LL	98102	99.47	92.24
LR	151	0.15	0.14

Quad

File: LOF ANN 18/11 POST.002
Sample ID:
Tube:
Acquisition Date: 19-Nov-09
Gated Events: 98631
X Parameter: FL4-H CD38 APC (Lo
Quad Location: 29, 40

Quad	Events	% Gated	% Tota
UL	409	0.41	0.38
UR	84207	85.38	79.21
LL	6832	6.93	6.43
LR	7183	7.28	6.76

File: LOF ANN 18/11 POST
Sample ID:
Tube:
Acquisition Date: 19-Nov-0
Gated Events: 98631
X Parameter: FL4-H CD38 A

Gate	Events	% Gated	%
G1	98631	100.00	
G2	8340	8.46	

% of CD34+/CD38 low cells

Fish analysis of whole bone marrow and CD34$^+$ selected population

Standard FISH analysis was performed in all 24 patients on at least 200 nuclei of whole BM cells and no Ph$^+$ cells were identified. When we consider the CD34$^+$ selected population, in 15/24 (63%) patients FISH analysis was performed on a median of 100 nuclei (range 100-300) while in 5/24 (21%) patients the median number of nuclei analyzed was 75 (range 60-86). In 4/24 (16%) patients FISH analysis was not performed due to the scarce yield of bone marrow purified CD34$^+$ and consecutively the inadequate number of evaluable nuclei (less than 50). [Tab. 3]

Tab. 3 CD34+ cells isolation and FISH analysis

TOT. PTS	24
Optimal CD34+ isolation and FISH analysis (≥ 100 nuclei analyzed, range 100-300)	15/24 (63%)
Sub-optimal CD34+ isolation and FISH analysis (median 75 nuclei analyzed, range 60-86)	5/24 (21%)
Inadequate CD34+ isolation (< 50 nuclei analyzed)	4/24 (16%)

We found residual CD34$^+$Ph$^+$ cells only in 1/20 (5%) evaluated patients. Of note, in this patient a total of 140 CD34$^+$ interphase nuclei were analyzed and only 1 of them was found BCR-ABL positive (0.7%). [Tab.2]

Early CD34$^+$Ph$^+$ evaluation during nilotinib treatment

In 5 additional patients we could evaluate CD34$^+$Ph$^+$ cells after only 3 months of front line nilotinib therapy. All 5 patients (3 males, 2 females, median age 59, range 43-67) were in CP at diagnosis and were enrolled in the CAMN107EIC01 study (EUDRACT code 2009-017775-19) receiving nilotinib 300mg/BID. After 3 months of nilotinib treatment conventional cytogenetics was performed and all 5 patients had achieved CCyR. At the same time FISH analysis on purified (median purity 87% -range 82-87%) CD34$^+$ cells was done and after evaluating a median of 100 nuclei (range 100-200) none of the 5 patients showed residual CD34$^+$Ph$^+$ cells. [Tab. 4]

Tab 4. "Early" $CD34^+Ph^+$ FISH evaluation

TOT PTS	5
Whole bone marrow Ph+ FISH analysis (300n)	0/5
Purified CD34+/Ph+ FISH analysis (median 100n, range 100-200n)	0/5

Patients' follow-up

Twenty-three out of 24 patients continued nilotinib and were monitored on a routine basis for their cytogenetic and molecular residual disease, while 1 patient switched to imatinib due to non hematological toxicity. After a median time of observation of 10 months (range 6-16 months) from $CD34^+Ph^+$ cells evaluation, all 23 nilotinib treated patients were still in CCyR. Regarding molecular response only 3/23 (13%) patients didn't achieve a MMolR, 11/23 patients (48%) were in MMolR with a median value of BCR-ABL/ABL ratio of 0.02% (range 0.01-0.05) and 9/23 (39%) patients were in CMolR. The only patient in which $CD34^+Ph^+$ progenitor cells were still detectable was in MMolR for 21 months at the time of $CD34^+$ evaluation and was in CMolR at the last follow-up.

DISCUSSION

For many CML patients, imatinib transforms a life-threatening disease into a chronic condition. Newly diagnosed patients with chronic phase CML have an almost 90% chance of being alive at 60 months after diagnosis[14]. However, most patients continue to test positive by RT-PCR, and disease recurrence upon discontinuation of drug is the rule even in the 60% of patients that become PCR undetectable[1]. This indicates that CML stem cells survive in the presence of imatinib and suggests lifelong continuation of therapy, with considerable economic expense and sometimes despite significant side effects. In this regard, during imatinib treatment, persistence of residual $CD34^+Ph^+$ progenitor cells has been documented *in vivo* even in patients with prolonged CCyR and MMolR[2,3,15]. Elucidating the mechanism by which persistent CML stem cells escape the effects of imatinib will be crucial for directing strategies to eradicate residual disease. The central question is whether disease persistence is BCR-ABL dependent, like many cases of resistance, or BCR-ABL independent. In the first scenario, overcoming disease persistence will require effectively targeting BCR-ABL in the relevant stem cell compartment.

The second generation TKI nilotinib may satisfy this requisition: in fact when compared to imatinib, front line nilotinib treatment induces a higher, earlier and deeper molecular response[6,7], yet *in vitro* data showed that nilotinib appeared not to be superior to imatinib in inducing growth inhibition of CML progenitor cells[9]. However, no data are available so far on the persistence of Ph^+ progenitor cells during nilotinib treatment in CML patients.

To explore this issue, we checked the presence of residual $CD34^+Ph^+$ cells in a series of patients treated with nilotinib since diagnosis and in stable CCyR. Surprisingly only 1/20 (5%) evaluable patients showed persistence of residual Ph^+ progenitor cells at a negligible level as just 1/140 (0.7%) $CD34^+$ interphase nuclei analyzed by FISH was found BCR-ABL positive. To our knowledge this is the first time that the efficacy of nilotinib in reducing leukemic $CD34^+$ cell burden has been evaluated *in vivo*. The results obtained in front line nilotinib treated patients are quite different from those we found previously in a fairly comparable series of CML patients in long lasting CCyR during imatinib treatment. In fact in the latter study about 45% of patients in CCyR for a median of 35 months, still harbored a median of 1% of $CD34^+Ph^+$ cells[3] [Tab. 5]. The difference we found (1/20 patients with

persisting $CD34^{+}Ph^{+}$ in the nilotinib group vs. 14/31 imatinib patients) is significant and it is even more remarkable considering that the median treatment length at the time of CML stem cell evaluation, was much longer in the imatinib patients cohort compared to the nilotinib one (39 vs. 22 months). We do not necessarily imply that nilotinib fully eradicates $CD34^{+}Ph^{+}$ cells: our data may suggest that enhanced BCR-ABL kinase inhibition displayed by nilotinib induces a "deeper" suppression of CML progenitors and that it may be necessary to analyze a much higher number of $CD34^{+}$ nuclei in order to reach a level of detectability. In addition, our study demonstrated that FISH analysis of residual $CD34^{+}Ph^{+}$ is feasible and could be considered an alternative method for the evaluation of minimal residual disease.

Tab. 5

	imatinib	nilotinib
N° of patients	31	20
median treatment duration (mos)	39	22
median CCyR duration (mos)	35	17.5
CD34+/Ph+ (%)	14/35 (45%)	1/20 (5%)
median % pos. Nuclei	1 (1-7)	n.a.

Our results are, apparently, in contrast to the recent data published by Kumari et al.[16] who showed that the number of BCR-ABL-positive precursors decreases significantly in all bone marrow compartments during MMolR. More importantly, they demonstrated substantially lower BCR-ABL expression levels in persisting MMolR colony-forming units (CFUs) compared with CML CFUs from diagnosis. Critically, lower BCR-ABL levels may indeed cause imatinib insensitivity, because primary murine bone marrow cells engineered to express low amounts of BCR-ABL were substantially less sensitive to imatinib than BCR-ABL-overexpressing cells, but were genetically more stable. Thus, MMolR is characterized by the persistence of CML clones with low BCR-ABL expression that may explain their insensitivity to imatinib and their low propensity to develop imatinib resistance through kinase point mutations. These results can be interpreted as follows: high level BCR-ABL expression is incompatible with persistence under imatinib, whereas low BCR-ABL levels contribute to intrinsic BCR-ABL kinase inhibitor resistance[17]. This suggestion

has an important implication: if low BCR-ABL expression is a hallmark of persisting stem and progenitor cells under kinase inhibitor therapy in vivo, 20-300 times more potent second generation BCR-ABL inhibitors such as nilotinib and dasatinib will not be presumably more potent in eradicating persistent CML and leukemia eradication concept should consequently target BCR-ABL independent pathways. On the other hand, we can suggest that if we achieve an earlier and stronger depletion of genetically less stable precursors expressing high BCR-ABL transcript with a more potent TKI, we could reduce the risk for emergence of secondary mutation and resistance.

In order to better characterize the "stemness" of $CD34^+$ purified cells, we studied the CD38 expression which identifies a more differentiated cell. We found that the majority of $CD34^+$ cells (86%) isolated from the bone marrow of nilotinib treated patients, was also $CD38^+$ with a high intensity of expression ($CD34^+CD38^{+high}$), while the rest of $CD34^+$ cells (14%) showed a low expression of CD38 antigen ($CD34^+CD38^{+low}$). The latter population, that is more undifferentiated and more quiescent, was also studied by FISH analysis but we cannot establish if the only $CD34^+Ph^+$ cell we identified belonged to this population. To obtain more detailed information about the characteristics of the residual leukemic stem cell during nilotinib treatment we should have the possibility of separating the two cell compartments ($CD34^+CD38^{+high}$ and $CD34^+CD38^{+low}$) and then performed FISH analysis in each subset of cells. However, due to poor cellularity of the bone marrow samples collected from nilotinib treated patients, it seems extremely difficult to isolate a number of $CD34^+CD38^{+low}$ cells sufficient to perform FISH analysis, unless collecting much larger amounts of bone marrow (about 50 ml), certainly more uncomfortable for the patient.

Regarding the correlation with molecular response, we found that the great majority of patients still presented residual disease at molecular level. The latter may be explained by the persistence, albeit "undetectable" of $CD34^+Ph^+$ cells and/or by the presence of more differentiated cells as source of molecular disease. However after a median of 10 additional months of treatment from $CD34^+Ph^+$ evaluation (i.e. a median of 32 months of nilotinib therapy from diagnosis) only 13% of patients didn't achieve MMolR, while 56% of them were in stable MMolR and 39% of them achieved CMolR.

In 5 additional patients we could evaluate CD34+Ph+ kinetic of reduction as they have been studied after 3 months only of nilotinib treatment. Surprisingly, together with achieving CCyR, all of them showed no detectable $CD34^{+}Ph^{+}$ cells. Despite the very limited number of patients studied, this may suggest that the fast inhibitory activity displayed by nilotinib on the bulk of CML affects precursor cells as well.

In conclusion, according to these preliminary results, the great majority of CML patients achieving CCyR with front line nilotinb treatment do not show, unlike imatinib treated patients in CCyR, persistence of $CD34^{+}Ph^{+}$ cells. Even if these data appear in line with the overall better clinical results observed with nilotinib, their significance in the path to cure of CML has still to be determined. If these findings will have an impact in the path to cure of CML with TKIs has still to be determined. We are aware that $CD34^{+}CD38^{+}Ph^{+}$ cells do not represent true quiescent stem cells but they identify a more differentiated precursor compartment which is indeed the real "fuel" of CML. Recent kinetic studies on leukemic and normal hematopoietic stem cells[18] revealed that early quiescent Ph+ stem cells maintain a dormant state, have a scarce tendency to divide and will enter the highly proliferating precursor state in a stochastic way: the latter may suggest that a marked dropping in CML precursor compartment obtained by second generation TKI nilotinib, could be sufficient to assure a long term disease remission. However additional data and a longer follow up are required to clarify if nilotinib is really more efficient than imatinib in eliminating CML progenitor cells in vivo and if this will translate in a significantly higher number of nilotinib treated patients achieving CMolR and able to discontinue the treatment without disease recurrence.

SUMMARY

Chronic myeloid leukemia originates in a hematopoietic stem cell with the reciprocal translocation t(9;22). The resulting Philadelphia chromosome produces BCR-ABL, a constitutively active tyrosine kinase that drives expansion of leukemic progeny. Target therapy with the TKI imatinib induces CCyR in more than 80% of newly diagnosed patients in chronic phase[14]. Most patients achieving CCyR, however, have BCR-ABL transcript detectable by PCR. Those whose disease is undetectable by RT-PCR usually experience recurrence of disease when imatinib is discontinued[1], which indicates that leukemic stem cells persist in most patients even when the disease burden is reduced below detectable limits. On this regard, during imatinib treatment, persistence of residual $CD34^+Ph^+$ progenitor cells has been documented in vivo even in patients with prolonged CCyR and MMolR[2,3,15].

The second generation TKI nilotinib induces, when compared to imatinib, a higher, earlier and deeper molecular response [6,7], but the in vitro data showed that nilotinib appeared not to be superior to imatinib in inducing growth inhibition of CML progenitor cells[9]. However, no data are available so far on the persistence of Ph^+ progenitor cells during nilotinib treatment in CML patients.

This study assessed for the first time the persistence of $CD34^+Ph^+$ precursor cells during nilotinib front line treatment. Our preliminary results showed that, in patients in CCyR, even after short term nilotinib therapy, residual leukemic progenitors are much rarely detected when compared to imatinib treated CCyR patients. If these findings will have an impact in the path to cure of CML with TKIs has still to be determined. In this regards recent kinetic studies on leukemic and normal hematopoietic stem cells revealed that early quiescent Ph+ stem cells maintain a dormant state, have a scarce tendency to divide and will enter the highly proliferating precursor state in a stochastic way: the latter may suggest that a marked dropping in CML precursor compartment obtained by second generation TKI nilotinib, could be sufficient to assure a long term disease remission even after nilotinib discontinuation.

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