University of Siena – Department of Medical Biotechnologies

Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC)

XXIX cycle (2013-2016)

Coordinator: Prof. Alessandra Renieri

Flow-cytometry evaluation of residual Leukemia Stem Cells in Chronic Myeloid Leukemia patients: feasibility and clinical application.

Scientific disciplinary sector: MED/15 – Hematology

Tutor

Prof.ssa Monica Bocchia

PhD Candidate

Dr.ssa Lara Aprile

Academic Year 2015/2016

Contents:

Abstract

Chronic Myeloid Leukemia (CML) is a myeloproliferative disorder characterized by the presence of the BCR-ABL1 oncogene, deriving from the t(9;22)-(q34;q11) balanced translocation. The deriving BCR-ABL1 fusion protein has tyrosine kinase activity, and it is capable of inducing the proliferation and expansion of myeloid precursors. Tyrosine kinase inhibitors, drugs capable of blocking the malignant proliferation of cells by directly targeting the BCR-ABL1 kinase activity, have dramatically changed patients's outcome and improved survival rate. Dormant CML-Leukemia Stem Cells (LSCs) are intrinsically refractory to TKIs and for this reason, only few patients can discontinue TKI therapy. CML LSCs are transcriptionally silent and probably not detectable by qRT-PCR. Several studies tried to find a specific marker able to detect CML LSCs and distinguish them from normal hematopoietic stem cells (HSCs). CD26 (dipeptidylpeptidase IV) was very recently identified as the most specific surface marker for flow-cytometry quantification and isolation of CML LSCs mainly in the bone marrow while rare samples of peripheral blood have been tested so far. Thus, principal aim of this study was to evaluate the presence of circulating LSCs in peripheral blood samples of patients during TKI therapy, avoiding in this way the need of routine bone marrow aspirate. Secondary we investigated a correlation, if any, between LSCs value and degree of molecular response. The analysis was conducted on 202 CML subjects. We have tested by flow-cytometry analysis the expression on peripheral blood cells of stem cells markers such as CD45, CD34, CD38 and, in particular, we have exploited the co-expression of the PE-conjugated anti-CD26. In our experiments we confirmed that CD26 is a specific marker able to discriminate normal HSCs from CML LSCs also in peripheral blood and that it is not expressed on LSCs of other myeloid disorders. The results of our study also showed that circulating CD26+ LSCs are easily detected in CML patients while on TKIs treatment and when comparing bone marrow and peripheral blood actual amount of CD26+ LSCs in the same patient, values were superimposable. However, no correlation was found between CD26+ LSCs count and BCR-ABL/ABL^{IS} ratio molecular analysis. This study assesses for the first time that flow-cytometry analysis of circulating CD45+/CD34+/CD38-/CD26+ LSCs is a powerful tool that could be of great help to identify the residual leukemic cells during follow-up of CML patients, just with routine peripheral blood testing. Further future investigations are required to establish possible roles of flow-cytometry LSCs detection

during management of CML patients and to evaluate a potential relationship between undetectable CML LSCs and safe treatment discontinuation.

Introduction

Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder characterized by the overproduction and accumulation of immature myeloid cells in the bone marrow, peripheral blood and spleen [1]. This increase of partially differentiated white blood cells, (i.e. myeloid precursors), is due to a specific acquired genetic alteration in the DNA of the Hematopoietic Stem Cells (HSCs). The malignant HSCs maintain the capability to self-renew and give rise to blood cells in a deregulated manner, behaving as disease-initiating Leukemic Stem Cells (LSCs) [2]. LSCs have a proliferative advantage and aberrant differentiation capacity over the normal counterpart and give rise to the expanded myeloid compartment [2].

The LSCs, as well as the whole progeny that arises from them, carry the Philadelphia (Ph) chromosome, resulting from a reciprocal balanced translocation, t (9;22)-(q34;q11), between the long arms of chromosomes 9 and 22 Fig.1 [3][4]. The molecular consequence of the new chromosomal combination is the expression of a fusion protein called BCR-ABL1, an oncogenic constitutively active tyrosine kinase [1]. In particularly, BCR-ABL1 results in the fusion of the *c-abl* oncogene 1 (ABL1) with the breakpoint cluster region gene (BCR) [1].

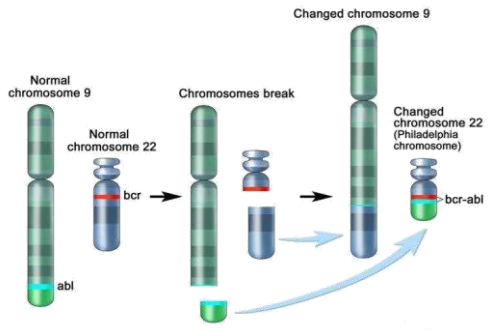

Figure 1. The t(9;22) (q34;q11) balanced translocation.

It is generally accepted that the acquisition of the BCR-ABL1 oncogene is the essential initiating event in the genesis of CML, the first disease in which such a specific genetic alteration inside cells has been directly linked to the malignant transformation [1-4]. BCR-ABL1 stimulates a number of key cell survival pathways to enhance proliferation and expansion of CML cells, inhibits cellular adhesion and retention

within the bone marrow niche, and reduces the possibility for Ph+ cells of dying by apoptosis [1-4]. The BCR-ABL tyrosine kinase is involved in many signaling pathways, such as PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and JAK/STAT [5,7].

In particular, BCR-ABL activity is responsible to:

1. Mitotic stimulus (RAS phosphorylation and the JAK-STAT system);
2. The interaction with adapter proteins, such as GBR2, and formation of multimeric complexes that will switch on these pathways;
3. The inactivation of several tumor suppressor genes, as p53, through regulation of gene expression, changing compartmentalization of cells or favoring proteins modifications trough phosphorylation/ubiquitination/acetylation events;
4. Genomic instability: the increased proliferation take from the checkpoint G1/S for the repair of DNA damage, the acquisition of additional genotype abnormalities is associated with the progression to accelerated phase and blast phase;
5. Inhibition of apoptosis (RAS phosphorylation) [5-7].

These mechanisms induce the uncontrolled expansion of myeloid progenitor cells, and their premature release into the peripheral blood as partially mature cells, due to the loss of balance between self-renewal and differentiation. Recent studies have suggested that other distinct molecular changes, acquired before or after the expression of BCR-ABL1, may also be required for disease onset, maintenance and progression, leading to a big heterogeneity in the treatment and prognosis of patients affected [5-8]. Indeed, CML can be somehow considered a stem “cell-derived but progenitor-driven disease”. LSCs give rise to a large number of Leukemia Progenitor Cells generally differentiate in more mature cells but, that can also start instead to self-renew and thus to give origin to blastic phase and disease progression [8]. The increase of progenitor cells reduces the number of stem cells and help the expansion of the neoplastic population, that is less responsive to the control by grow-regulatory signal of cytokines and bone marrow microenvironment. Contemporary, the immature blasts also lose adherence to marrow stromal, an event that help their mobilization to peripheral blood, and develop antiapoptotic mechanisms to avoid death. The majority of additional cytogenetic and molecular changes, that may be found in these CML cells, occur in 50 to 80 percent of

patients during the transition from chronic to the accelerated and blastic phase, and help the fastest progression of the disease [8].

CML treatment: between great success and new challenges.

Tyrosine kinase inhibitors

The introduction of imatinib, and subsequent tyrosine kinase inhibitors (TKIs), for the treatment of CML in the early chronic phase has been one of the great success stories of modern medicine. Imatinib-mesylate (Glivec®-Novartis, STI571) is the first tyrosine kinase inhibitor (TKI) developed in 1999 for the treatment of chronic phase CML patients and it is currently used as a first line therapy. First imatinib success was documented in IRIS study a phase 3 international randomized study who compared interferon plus cytarabine with imatinib. Patients randomized to the TKI gained a significant improvement in progression-free survival compared with patients randomized to interferon plus cytarabine with durable response as shown in a 6 year follow-up of the study [9,10]. In fact, estimated event free survival rate was 88%, and OS rate was 95% when only CML-related deaths were considered [10].

Second and third generation TKIs (nilotinib, dasatinib, bosutinib and ponatinib) confirmed and exceed imatinib results both in first line than in second line of treatment after failure or intolerance of a previously TKI [11-14]. Consequently, the natural history of CML has been dramatically changed and while in the past patients might have died of progressive disease within few years from diagnosis, now are more likely to die of other causes than from CML [15].

Monitoring of disease

A deep knowledge of the biological and molecular basis of this disease, together with the development of TKIs that cause an almost complete quiescence, led to the development of increasingly sophisticated techniques and parameters to evaluate the course of CML. Furthermore, the goal of CML patients' management changed over the years. Clinical responses are divided into hematologic, cytogenetic and molecular. Hematologic response is identified with the normalization of blood parameters. Cytogenetic response evaluates the percentage of Ph+ cells surviving during therapy, it is performed by conventional cytogenetic test on 20 bone marrow metaphases, or by molecular cytogenetics (FISH) on 200 nuclei [16]. The definition of molecular response

is based on the log reduction in the BCR-ABL1 transcripts identified by Quantitative Reverse Transcription polymerase chain reaction (RQ-PCR). This assay allows us to know the amount of pathological BCR-ABL transcript compared to the ABL control and therefore it is a precise analysis of the amount of residual transcriptionally active disease. While during the first years of "TKIs era" clinicians and CML patients aimed for a complete cytogenetic and/or major molecular response (BCR-ABL/ABL < 0.1, MMR, MR3), the modern goal in the management of CML is a deep molecular response (BCR-ABL/ABL < 0.01-0.0032, MR4, MR4.5) Figure2.

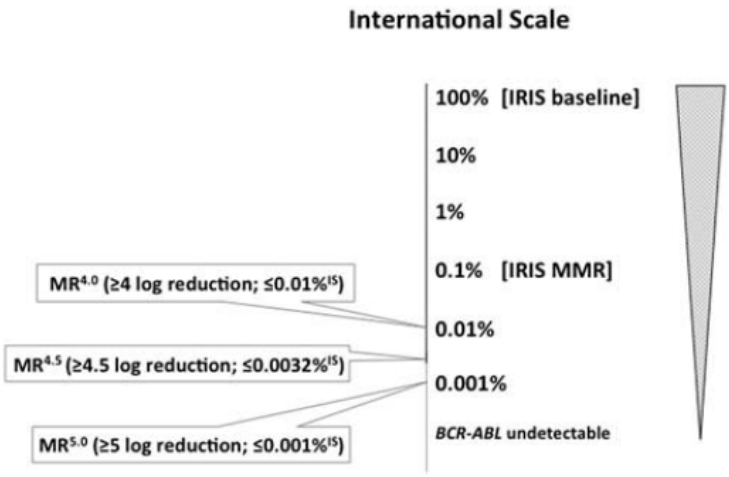

Figure 2. molecular response according to International Scale

In the first 6 to 12 months of treatment many patients achieve MR3, but with a continuing TKI treatment a large fraction of patients will have a further reduction in the level of molecular residual disease, leading to progressively deeper molecular response [17]. MR4.5 occurs in 20% of imatinib treated patients in the first 2-3 years, but this proportion rises to 40% after 5 to 7 years [17]. Using more potent TKIs, such as nilotinib or dasatinib, the rates of MR 4.5 in the first 2 years are higher [18,19]. In the phase 3 ENESTnd clinical study that compared nilotinib 300 mg twice daily with imatinib 400 mg daily as first line therapy, the rate of MR4.5 by 4 years was 40% in nilotinib cohort vs 23% in the imatinib ones [17]. In the dasatinib vs imatinib study (DASISION) MR4.5 was achieved by 3 years in 22% vs 12% of patients respectively [19].

Future direction: treatment free remission

We can conclude that for most CML patients, the advent of TKIs therapy has turned their disease into an indolent medical condition. Even if the survival rate has improved a lot, to avoid risks of relapse patients need to continue taking TKIs, without stopping the treatment ever. Assuming that the incidence of CML is relatively constant, improving survival rate leads to increasing prevalence of this disease. According to epidemiological data more than 400.000 CML patients expected in Europe by the year 2050 with a consequent significant burden on health care system due to the cost of a lifelong treatment [20]. Furthermore even if TKIs are generally well tolerate a proportion of patients suffer from several and previously not yet known side effects [21,22] . In this scenario, the possibility to stop TKIs in a selective group of patients is a very attractive and cost effective way to optimize available therapy. On the other end, the question remains open about the possibility to achieve a TKI-and-leukemia free state called Treatment Free Remission (TFR), in patients with chronic phase CML that achieve a deep and durable response and could stop TKIs [23]. Until now, several "discontinuation" studies have been performed. In the majority of them, study population was characterized by patients with a stable MR4 or MR4.5. Even in this very selected setting of patients, withdrawing of TKIs was followed by a molecular relapse in about 60% of them [24-27]. The largest discontinuation study to date is the European Stop Kinase Inhibitor (EURO-SKI) trial of the European Leukemia Net. This study has recruited 821 chronic phase CML patients in 11 different European countries. Molecular recurrence-free survival was 62% at 6 months, 56% at 12 month and 52% at 24 month on an "Intention to Treat Basis". No progression to advanced disease phase was documented and patients who lost molecular response regained a deep molecular response re-starting previous therapy (mainly imatinib) [26]. Limited experience is about TFR after second generation TKIs. During last ASCO (American Society of Clinical Oncology) annual meeting Hochhaus et al presented data about ENEST-freedom study, the first one to specifically investigate TFR following frontline nilotinib therapy. Patients with chronic phase CML treated with nilotinib for more than 2 years and with MR 4.5 at screening were enrolled. Upon enrollment, after 1 year of "consolidation" nilotinib treatment patients with no molecular assessment worse than MR 4, were eligible to stop treatment (190/250 patients). Loss of MR3 triggered re-initiation of nilotinib. At week 48 of the TFR phase, 51.6% of 190 patients were in MR3 and had not re-initiated treatment [27].

TKI discontinuation: LSCs role

Despite consolidated clinical data coming from discontinuation study, the reason why only a minority of CML patients in deep molecular response can safety discontinued TKI therapy is not yet clear. Available data suggest that relapse after TKIs discontinuation is due to the persistence of LSCs intrinsically resistant to TKIs [28]. Dormant LSCs, are generally slow cycling or even quiescent, for this reason, they are intrinsically refractory to TKIs, drugs that work instead on active cells having a high metabolism and cell cycle [29]. Furthermore, recently, it has been suggested that CML LSCs, on the contrary to CML progenitors cells, do not express BCR-ABL1 mRNA and their survival is independent of BCR-ABL1 due to the activation of several pathways BCR-ABL1 independent [28-32].

LSCs identification

If CML LSCs do not or very low express BCR-ABL1 mRNA, qRT-PCR, the most sensitive assay to monitor disease status in CML patients, may be inappropriate to quantify residual quiescent CML LSCs that are transcriptionally silent. The best chance to treat and monitor CML patients, and define those resistant to therapy, would be that of easily identifying residual LSCs, and many researches have focused on their identification and separation from the normal HSCs. This represents a big challenge because during TKIs therapy the number of residual LSCs may be so low and overgrown by normal stem cells. A big help has come by the use of flow- cytometry, a technique that permits the detection of aberrant cell surface markers, that could characterize the malignant clonal populations compared to normal stem cells, through the use of specific labelled monoclonal antibodies. In chronic phase CML patients, BCR-ABL1+ LSCs resides as well as normal HSCs within the CD45+/CD34+/CD38-/Lin- fraction. The first attempts to isolate these CD34+ Ph+ cells have been made by separating, according to different study CD34+/CD38- or CD34+/CD38+ bone marrow cells of CML patients by the use of immune magnetic beads, with subsequent BCR-ABL FISH analysis of the separated population [33-35]. After positive selection, flow-cytometry was used to assess the purity of the sorted CD34+ cells. Through this sorting method, it has been demonstrated that CD34+/Ph+ cells could be still detected in a high percentage of patients with sustained and prolonged Complete Cytogenetic Response

during treatment with TKIs, both imatinib than second generation TKIs, explaining the high risks of disease recurrence after discontinuation of these drugs [33-35]. However, this method of CD34+ isolation by immune magnetic beads and FISH analysis has 1) low sensitivity (the yield of CD34+ after beads elution is extremely low) 2) low specificity (the CD34+/CD38+ fraction includes CML precursors while the CD34+/CD38- potentially comprises normal HSCs) and 3) it is very time consuming. Additionally, it requires undergoing a bone marrow aspirate as routine follow up for CML patients, a procedure that it is today anachronistic in this setting of patients.

Nowadays, to make more accurate our analysis we have instead the possibility of evaluating the leukemic stem cells by multicolor flow-cytometry and characterize them by the co-expression CD45+/CD34+/CD38- cells and additional specific markers. In fact, to better discriminate CML LSCs from HSCs, that reside in the same CD34+/CD38- fraction, many others surface antigens have been proposed. Indeed, CML LSCs aberrantly express the IL-1 receptor accessory protein, IL-1RAP, and higher levels of Siglec-3 (CD33) and interleukin-3 receptor alpha chain (IL-3RA = CD123) than HSCs [36-38]. They also express several surface antigens that seem to be relevant for HSC-niche interaction, like CD44 and CXCR4, but, compared to HSCs that are highly attracted to the niche by the stroma cell- derived factor-1 (SDF-1) binding to CXCR4, LSCs seem to escape this chemotactic effect not responding to SDF-1 and being capable in this way of exiting the niche [39,40]. Other additional markers are IL-2RA (CD25), the cell surface target antigen campath-1 (CD52) and, most importantly, the cytokine-targeting surface enzyme dipeptidyl-peptidase IV (DPPIV) (CD26), that seems to be today the most specific marker for CML LSCs (Table1) [41,42].

Table 1 Expression of cell surface markers and targets on LSCs in CML and AML

	Expressed on CD34⁺/CD38⁻ cells			
Target/Marker	CD	CML LSCs	AML LSCs	Normal stem cells
IL-2RA	CD25	+	+/–	–
DPPIV	CD26	+	–	–
Siglec-3	CD33	+	+	+/–
Pgp-1	CD44	+	+	+
IAP	CD47	+	+	+/–
Campath-1	CD52	+	+	+/–
Thy-1	CD90	+	–/+	+
Tactile	CD96	–	+/–	–
G-CSFR	CD114	+	+/–	+
KIT/SCFR	CD117	+	+	+
AC133	CD133	+	+/–	+
CXCR4	CD184	+	+	+
IL-1RAP	n.c.	+	+/–	–/+
CLL1	n.c.	–/+	+	–/+

According to recent studies, CD26 expression is restricted essentially to CD34+/CD38- LSC in almost all patients with newly diagnosed CML [41,42]. It is a marker detectable in CML but not on stem cells of other myeloid neoplasms, with the exception of very few AML patients [42]. As determined by cell sorting and FISH analysis, while 100% of purified CD26+ cells could express BCR-ABL1 at FISH and RT-PCR, the CD26- stem cells fraction was found BCR/ABL1 negative, demonstrating the high specificity of CD26+ as a reliable and robust biomarker of CML LSCs [42]. DPPIV is a cell surface antigen with enzyme activity that regulates the cytokine metabolism in the HSC-niche by a proteolytic degradation of various cytokine ligands, including IL-3, granulocyte-macrophage colony-stimulating factor and SDF-1 (stroma-derived factor-1). The degradation of SDF-1 to inactive fragments helps LSCs mobilization, because the inactive SDF-1 could not attract these cells to the niche through CXCR4. The disrupted SDF-1-CXCR4 interaction helps enhancing the number of LSCs that escape from bone marrow to blood [41,42]. Furthermore, CD26 expression is an early BCR-ABL1 independent event in leukemogenesis that may be critical for disease initiation and/or manifestation, while, in a terminal stage of the disease, CD26 may no longer be required to trigger the spread and expansion of LSC, since patients in blast phase had CD26- cells. Scientists have also tried to correlate CD26 expression with disease activity: while patients with Complete Cytogenetic Response and/or MR3

had undetectable bone marrow CD34+/CD38-/CD26+ cells, suggesting that most CML LSCs cells have been suppressed or depleted, in patients with relapsing CML or resistance disease at least a sub fraction of cells was still CD26+. This demonstrated that CD26 could be used as follow up parameter to monitor the presence and number of CML LSCs in patients resistant to TKIs [41]. Furthermore, Culen et al. quantified CD26+ LSCs bone marrow compartment in 31 CML patients at diagnosis and asked whether the number of CD26+ correlates with CML clinical parameters or with subsequent treatment response [43]. No correlation was found between the number of CD26+ CML-LSCs and Sokal, Hasford, or EUTOS risk stratification, while a correlation was found with time to achievement of MR3 at 12 month even if was not confirmed at 18 month [43]. Thus CD26, more than other previously identified stem cell markers such as IL1-RAP, CD25 and CD90 emerged as a potential biomarker for the quantification and isolation of CML LSCs, in bone marrow samples of CML patients [41].

Aim of the study

Until now, attempts to measure residual CML LSCs were based on separation of CD34+/CD38- bone marrow cells by immune magnetic beads, with subsequent BCR-ABL1 FISH analysis of the separated population. This method has low specificity and sensitivity and it is time consuming. Additionally, the need to undergo a bone marrow aspirate as routine follow up for CML patients it is nowadays anachronistic. The possibility to easily quantify residual LSCs in CML would represent a real challenge for this disease. On one hand, we could identify potentially "cured" patients in which we could safely stop TKI treatment. On the other hand, the capability to easily detect and sort CML LSCs would offer a great tool to test new drugs able to eliminate this subset.

Flow-cytometry, given the presence of appropriate specific markers, is an ideal technique for the identification of cellular subpopulations, of both normal and leukemic cells, in whole peripheral blood and bone marrow samples. Moreover, we have now the possibility of detecting from 8 up to 10 different simultaneous fluorescent signals, with high accuracy for cells identification even in case of small size populations and with a reduction time for acquisition and analysis.

Aims of the present study were:

1. To explore the feasibility and specificity of detecting circulating LSCs in the peripheral blood of CML patients, by using flow-cytometry and determining co-expression of dipeptidylpeptidase IV(DPPIV5CD26) in the CD34+CD38- fraction. Differently from the previous works [41,42], focused on bone marrow LSCs detection in newly diagnosed chronic phase CML patients, we will focus the attention on chronic phase CML patients in first-line TKIs treatment.
2. To search a correlation, if any, between number of residual LSCs and molecular response (BCR-ABL/ABL^{IS} ratio).

Materials and methods

Patients population and samples collection

CML patients during first line treatment with any approved TKI, referring to several Italian Hematology Centers entered this not interventional cross sectional study after signing a proper informed consent. Six mls EDTA peripheral blood samples were collected and centralized in Siena during a routine follow up visit. In 5 patients at the time of blood sample a bone marrow aspirate was also taken. All peripheral blood RT-PCR analysis have been performed at the same time of LSCs evaluation to monitor molecular responses of patients.

Immunophenotyping

The immunophenotyping study, for the identification of surface antigens, requires first of all, the evaluation of blood samples cell concentration. In order to obtain a concentration of 1 x 10^6/ml of cells for the analysis, different tubes for each patient were prepared with 200 µl of peripheral blood in each. The cell suspensions were then lysed using ammonium chloride at concentration 1:10. One hundred µl of lysed sample were incubated for 15 minutes at room temperature in the dark with a combination of 4 fluorophore-labelled monoclonal antibodies at the concentrations provided by the manufacturer, and including V500 conjugated anti-CD45 (BD Biosciences), FITC conjugated anti-CD34 (581), APC conjugated anti-CD38 (HIT2) and PE conjugated anti-CD26 (M-A261). Isotype controls were also included in the staining not containing the PE conjugated anti-CD26. The samples were then washed with PBS, and once resuspended they were ready for the analysis by flow-cytometry. The number of total cells acquired for each sample was around 1.000.000, so to obtain a sensitivity between 10^{-5} /10^{-6} and have the future possibility to compare the events with the data obtained by BCR-ABL1 RT-PCR evaluation.

To validate our procedure, we performed:

1. A comparison of flow-cytometry analysis results between bone marrow and peripheral blood for patients at diagnosis in which both samples were available;
2. A BCR-ABL1 FISH analysis of purified CD45+/CD34+/CD38-/CD26+ and of CD45+/CD34+/CD38-/CD26- after sorting procedure;
3. A CD45+/CD34+/CD38-/CD26+ flow-cytometry analysis in patients affected by hematological malignancies other than CML.

Statistical Analysis

According to the study variables, the cohort size calculation was determined by using PSS statistical analysis (SAS Power and Sample Size) with a power of 90% (associated with a $p < 0.01$) considering the vast possibilities of values undetermined for stem cells and molecular analysis. According to this analysis, the cohort size were 36 subjects.

The measurable variables (expressed as SEM ± SD where applicable) are evaluated by Mann-Whitney U test and the Kendall-test for possible linear correlations between them, accepting a $p < 0.05$ as significant value. All calculations were performed using SPSS (version 13).

Results

Validation of flow-cytometric procedure

Bone marrow vs peripheral blood evaluation

To validate our procedure, we performed flow-cytometry analysis in 5 newly diagnosed CML patients simultaneously in bone marrow aspirate and in peripheral blood. The median percentage of CD26+ cells within the CD34+/CD38- fraction was 29,7 (range 16,4-84,42 %) in bone marrow samples and 43,7 (range 22,31-76,31%) in peripheral blood samples. However as reported in Table 2, the median absolute number of CD26/µl appeared superimposable in bone marrow and peripheral blood samples (22,76 and 17,41; range 3,4-83,72 and 3,51-167,04 respectively) thus making feasible and reliable to quantify LSCs directly from peripheral blood samples.

	Median % of CD26+ within CD34+/CD38- fraction	**range**	**Median CD26+/µl**	**range**
Bone marrow	29,7	16,4-84,42	22,76	3,4-83,72
Peripheral blood	43,7	22,31-76,31	17,41	3,51-167,04

Table 2. Comparison between bone marrow and peripheral blood CD26+ LSCs

FISH analysis of selected LSCs

According to our hypothesis and to some extent to the literature CD34+/CD38-/CD26+ cells represent CML-LSCs potentially characterized by a Ph chromosome transcriptionally silent. To prove the assumption and confirm the clonal origin of CD26+ population we decided to perform a FISH analysis of both peripheral blood sorted CD34+/CD38-/CD26+ and CD34+/CD38-/CD26-. To do so, we choose 5 patients with peripheral blood LSCs count > 0.01/µl adequate to perform a fluorescence activated cell sorting (FACS). LSCs range count was 0.0175 – 0.12 /µl and 50 ml of peripheral blood were necessary for the assays. After FACS a median of 300 CD34+/CD38- cells were sorted (range 900-25), a median of 7 of these were also CD26+ (range 10-2), the other ones were CD26-. As well as previously reported in bone marrow experiments, [42] in all 5 analyzed peripheral blood samples, almost all

cells in the CD26+ fraction were BCR-ABL1+ by FISH (Figure 3). On the contrary no BCR-ABL+ cells were found in the CD34+/CD38-/CD26- sorted population.

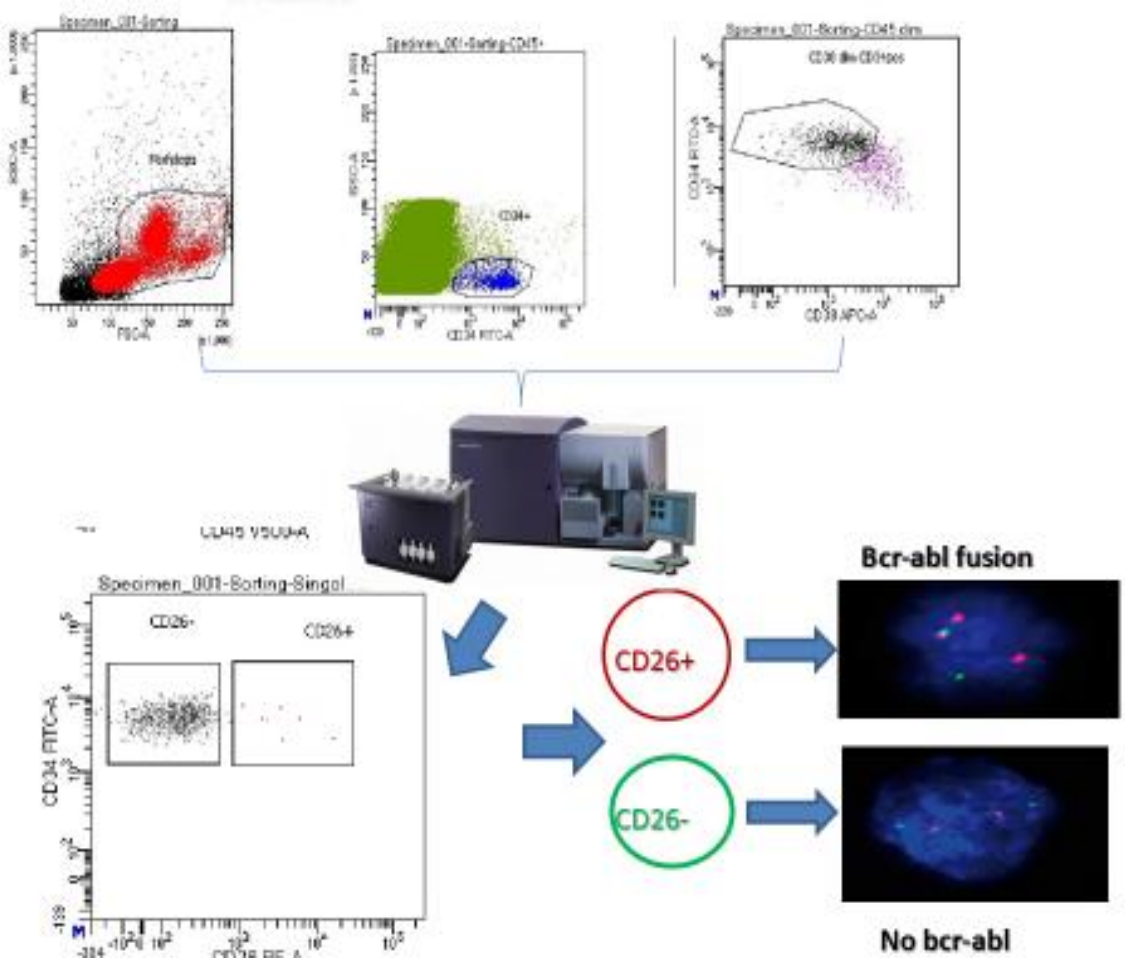

Figure 3. Sorting and FISH analysis of selected CD45+/CD34+/CD38-/CD26+ and CD45+/CD34+/CD38-/CD26- cells

Evaluation of CD26 marker in stem cells of other hematological disorders

In order to demonstrate the specificity of CD26 marker for CML LSCs, we have tested for the presence of circulating CD45+/CD34+/CD38-/CD26+ cells several samples of patients affected by other myeloid disorders, such as acute myeloid leukemia (AML, 7 samples) or myelofibrosis (MFI, 10 samples), or of patients undergoing granulocyte colony stimulation factor (GCSF) treatment, after a chemotherapeutic regimen (5 samples). Despite the presence of variable amounts of CD45+/CD34+/CD38-, the expression of CD26 in this subpopulation was never

detected. Figure 4 shows a representative multiparametric flow-cytometry analysis of a MFI patient. As stated above, despite an increased number of circulating CD45+/CD34+/CD38-, these cells do not express the CD26 marker.

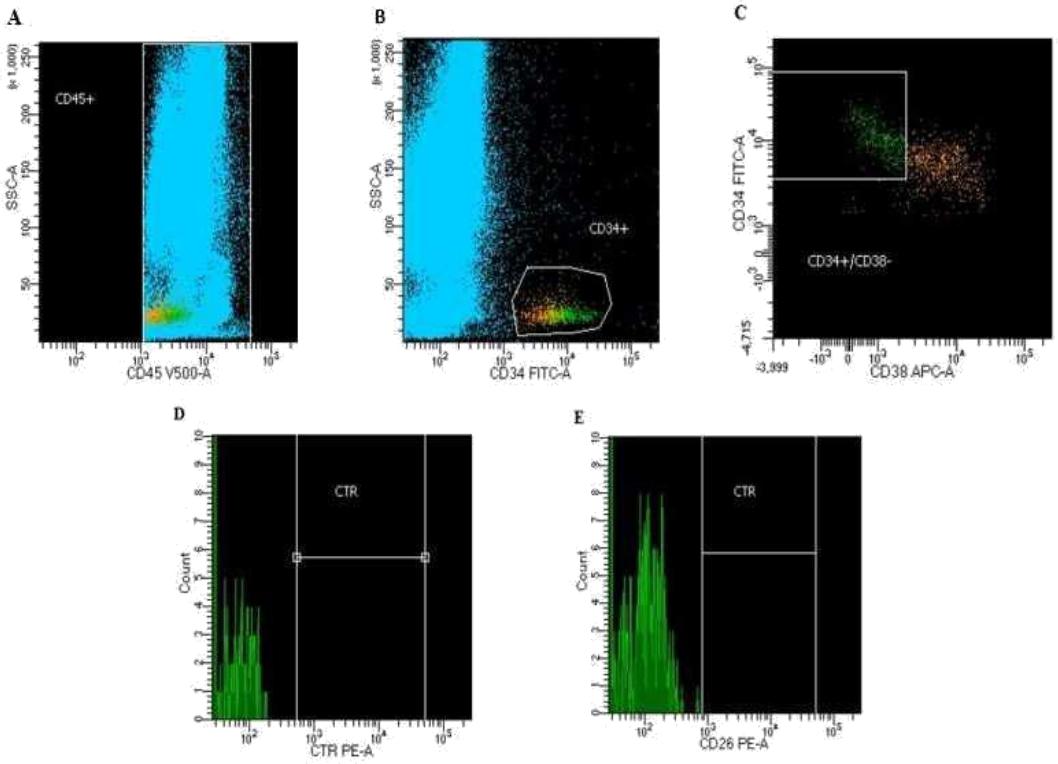

Figure 4. Multiparametric flow-cytometry analysis of MFI negative control. (A) Circulating leucocytes identified by CD45+ (blue, green and orange). (B) Circulating CD34+ cells (green and orange). (C) Expression of CD38 on CD34+ circulating cells. (D) Negative control and (E) CD26 expression on CD34+CD38-

Patients clinical data

Flow-cytometry evaluation of circulating LSCs has been made on 202 CML patients, after collecting peripheral blood samples from 10 different Italian Hematologic centers from July 2015 to December 2016. As shown in Table 3, patients were at different time of treatment with any of the 3 available TKIs approved for first line. Ninety-nine imatinib treated patients were on treatment for a median time of 79 months (range, 1-175 months); 81 patients were on nilotinib treatment for a median time of 24

months (range, 1-132 months) and 22 dasatinib treated patients were on treatment for a median time of 8,5 months (range, 1-107 months).

	Total patients	**IMATINIB treated patients**	**NILOTINIB treated patients**	**DASATINIB treated patients**
N°	202	99	81	22
Median TKI duration (month)	39 (1-175)	79 (1-175)	24 (1-132)	8,5 (1-107)
Median BCR-ABL1/ABL1^{IS} ratio (range)	0.004 (0-61)	0,0042 (0-61)	0,007 (0-11,7)	0,015 (0-1,39)
Median n° of CD26+/ µl (range)	0,0165 (0,0018-0,66)	0,014 (0,0029-0,24)	0,0208 (0,0035-0,66)	0,0099 (0,0018-0,09)
Patients with undetectable peripheral blood LSCs (%)	56/202 (27,7)	28/99 (28,7)	23/81 (28,3)	5/22 (22,7)

Table 3. LSCs evaluation during first-line TKIs therapy.

Evaluation of LSCs

Flow-cytometry CML LSCs evaluation was feasible in all 202 CML patients as all centralized samples met the appropriate criteria of cells viability and number. Figure 5 shows a representative flow-cytometry analysis of peripheral blood samples from CML patients on TKIs treatment. CD26+ LSCs were detected in 146/202 patients with a median value of 0,0165 µl (range, 0.0018-0.66 µl). 56/202 had no detectable CD26+ LSCs. Considering patients according to different TKI therapy, CD26+ LSCs were detectable in 71/99 imatinib treated patients, in 58/81 nilotinib treated patient and in 17/22 patients dasatinib treated. Median values are described in Table 3.

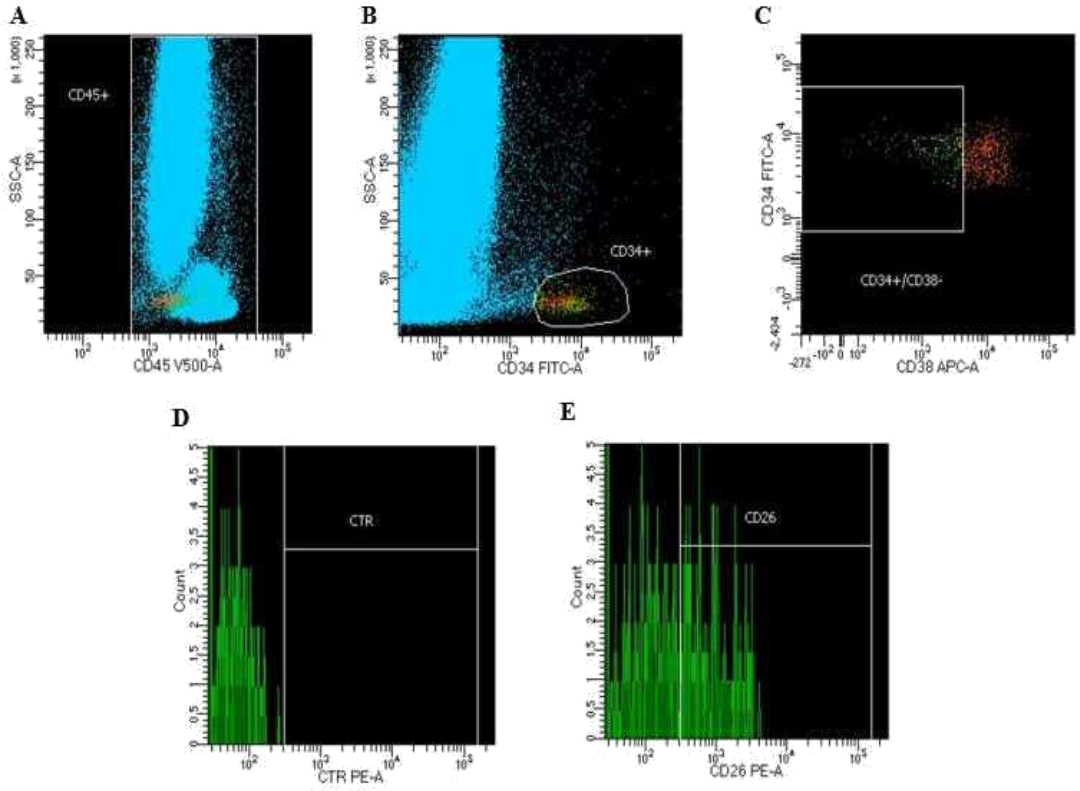

Figure 5. Multiparametric flow-cytometry analysis of CML samples. (A) Circulating leucocytes identified by CD45+ (blue, green and orange). (B) Circulating CD34+ cells (green and orange). (C) Expression of CD38 on CD34+ circulating cells, in yellow LSCs. (D) Negative control and (E) CD26 expression on CD34+CD38-

LSCs and molecular response

All 202 CML patients evaluated for CML LSCs were simultaneously tested for routine BCR-ABL/ABL^{IS} ratio. In 146/202 samples with measurable CD26+ LSCs BCR-ABL/ABL^{IS} ratio showed a median value of 0,004 (range 0-61). Kendall rank correlation coefficient used to analyze the relation between the measurable variables showed no correlation between BCR-ABL/ABL^{IS} ratio and number of residual CD26+ LSCs (r 0.118 p=0.097). In 56/202 patients CD26+ LSCs were undetectable, yet we found no correlation with the concomitant degree of molecular response.

Discussion

This study assesses for the first time that flow-cytometry analysis of circulating CD45+/CD34+/CD38-/CD26+ LSCs is a powerful tool that could be of great help to identify residual leukemic cells during follow-up of CML patients, just with routine peripheral blood testing.

Treatment of CML, a myeloproliferative stem cell disorder characterized by the presence of BCR-ABL1 oncogene, has changed a lot during the last decades and the introduction of selective TKIs has changed patients' outcome and, consequently, management of CML. First-line treatments with imatinib, nilotinib or dasatinib have all contributed to fulfill a longer median overall survival of CML patients and a lower rate of progression [10-12]. For many years since their appearance in the treatment scenario of CML, TKIs have been considered an extraordinary highly effective life-long treatment with a consequent significant burden on health care system [20]. More recently, several studies have demonstrated that a fraction of CML patients may stop TKIs treatment without recurrence of disease [24-27]. The achievement of a deep and sustained molecular response is mandatory for attempting TFR, yet approximately 40% to 60% of patients with a deep molecular response stopping TKI will lose the response and require retreatment [24-27]. Relapse after TKIs discontinuation is a phenomenon that has explained by LSCs intrinsic resistance to TKIs [28]. Survival of CML LSCs may be the consequence of the activation of BCR-ABL1 independent pathways [28-32]. As such, qRT-PCR, the most sensitive assay to monitor disease status in CML patients, may be inappropriate to quantify residual quiescent CML LSCs potentially transcriptionally silent. Previous attempts to detect and quantify CML LSCs have been made with immune magnetic beads based positive selection and subsequent FISH analysis [33-35]. However, this method has a low sensitivity, it is highly cost effective and not suitable for a routine LSCs evaluation.

LSCs supposedly reside within the CD34+/CD38-/Lin- fraction of the leukemic clone, but this antigens combination characterizes also normal hematopoietic stem cells. Additional markers are required to discriminate CML LSC from normal stem cells. Several surface markers such as CD123, CD25, CD117, IL1RAP, ST2, CD26 have been recently proposed to better characterized CML LSCs, but few have so far been proven to distinguish Ph positive from Ph negative LSCs [36-40]. Recently, Valent et al described that bone marrow CD34+/CD38-/Lin- CML LSCs specifically co-express

DPPIV (CD26) and that the expression of CD26 appears to discriminate CML LSCs from normal HSCs [41,42]. Based on these data, CD26 appears to be a potential biomarker for the quantification and isolation of CML LSCs at least in bone marrow aspirates [43]. However, the monitoring of residual LSCs in the bone marrow it is anachronistic to propose to CML patients today. For this reason, the possibility to test and optimize an easy system for CML LSCs detection it is a challenge for the next future.

In this study, our main goal was to demonstrate the possibility to detect with flow-cytometry analysis LSCs in CML patients by assessing peripheral blood samples. Our data demonstrate that CD26+ LSCs are Ph+, circulate in the blood and are easily detected and quantified by flow-cytometry in a reproducible, reliable and disease-specific manner. Our results are robust, as the analysis has been conducted on 202 follow-up patients, undergoing treatment with three different first-line TKIs, imatinib, nilotinib and dasatinib, at different time of therapy. We demonstrated in more than 70% of patients on a routine follow-up visit, the persistence of residual CD26+ LSCs with the advantage, by working directly on peripheral blood samples, to avoid the need of bone marrow aspirate. Indeed, the comparison between CD26+ absolute counts in bone marrow vs peripheral blood in 5 cases of newly diagnosed chronic phase CML patients revealed a superimposable absolute count. All 202 CML patients were evaluated also for molecular response (BCR-ABL/ABL^{IS} ratio) according to clinical practice. No correlation was found between the number of CD26+ LSCs and degree of molecular response. This evidence, even if determined by a cross sectional and not a prospective analysis, suggests that the molecular response refers to transcriptionally active CML progenitor cells and not to quiescent, TKIs resistant, CML LSCs.

In conclusion, the data collected in this thesis represent the first systematic evaluation of circulating LSCs in peripheral blood of CML patients.

Further investigations are required to achieve a more complete understanding about the role flow-cytometry could acquire in the clinic for the evaluation of LSCs in CML patients, both at diagnosis and during follow up. We think that the possibility to monitor easily peripheral blood CML stem cell burden will furnish the bases for clinical studies investigating the behavior of CML LSCs during treatment, mainly focusing on the still unknown correlation between residual stem cells and molecular response with the ultimate goal of identifying those patients optimal candidates for a safe TKI

discontinuation. For these reasons, we are conducting prospective studies with the intent to evaluate the behavior of peripheral blood CML LSCs in patients during different TKIs treatment, and in patients that discontinued TKIs treatment.

References

1. Faderl, S., Talpaz, M., Estrov, Z., et al. The biology of chronic myeloid leukemia. New England Journal of Medicine, 1999. 341(3), 164-172.

2. Passegué, E., Jamieson, C. H., Ailles, L. E. et al. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proceedings of the National Academy of Sciences, 2003.100 (suppl 1), 11842-11849.

3. Nowell, P. C., Hungerford, D. A. Chromosome studies on normal and leukemic human leukocytes. Journal of the National Cancer Institute, 1960. 25(1), 85-109.

4. Rowley, J. D. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature, 1973243:290–293.

5. Shet AS, Jahagirdar BN, Verfaillie CM. Chronic myelogenous leukemia: mechanisms underlying disease progression. Leukemia. 2002; 16:1402-411.

6. Di Bacco A, Keeshan K, McKenna SL, et al. Molecular abnormalities in chronic myeloid leukemia: deregulation of cell growth and apoptosis. Oncologist. 2000; 5:405-15.

7. Crivellaro, S., Carrà, G., Panuzzo, C., et al. The non-genomic loss of function of tumor suppressors: an essential role in the pathogenesis of chronic myeloid leukemia chronic phase. BMC cancer, 2016. 16(1), 1.

8. Skorski, T. Chronic myeloid leukemia cells refractory/resistant to tyrosine kinase inhibitors are genetically unstable and may cause relapse and malignant progression to the terminal disease state. Leukemia & lymphoma, 2011. 52(sup1), 23-29.

9. O'Brien SG, Guilhot F, Larson RA et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med. 2003. 13;348(11):994-1004.

10. Hochhaus A, O'Brien SG, Guilhot F et al. Six-year follow-up of patients receiving imatinib for the first-line treatment of chronic myeloid leukemia. Leukemia. 2009 23(6):1054-61.

11. Saglio G, Kim DW, Issaragrisil S, et al; ENESTnd Investigators. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. N Engl J Med. 2010. 362:2251-9.

12. Cortes JE, Saglio G, Kantarjian HM, et al. Final 5-Year Study Results of DASISION: The Dasatinib Versus Imatinib Study in Treatment-Naïve Chronic Myeloid Leukemia Patients Trial. J Clin Oncol. 2016 Jul 10;34(20):2333-40.
13. Poch Martell M, Sibai H, Deotare U, et al. Ponatinib in the therapy of chronic myeloid leukemia. Expert Rev Hematol. 2016. 9(10):923-32.
14. Cortes JE, Khoury HJ, Kantarjian HM, et al. Long-term bosutinib for chronic phase chronic myeloid leukemia after failure of imatinib plus dasatinib and/or nilotinib. Am J Hematol. 2016. 91(12):1206-1214.
15. Brunner AM, Campigotto F, Sadrzadeh H et al. Trends in all-cause mortality among patients with chronic myeloid leukemia: a Surveillance, Epidemiology, and End Results database analysis. Cancer. 2013. 15;119(14):2620-9
16. Testoni N, Marzocchi G, Luatti S, et al. Chronic myeloid leukemia: a prospective comparison of interphase fluorescence in situ hybridization and chromosome banding analysis for the definition of complete cytogenetic response: a study of the GIMEMA CML WP. Blood. 2009;114:4939-943.
17. Branford S1, Seymour JF, Grigg A, et al. BCR-ABL messenger RNA levels continue to decline in patients with chronic phase chronic myeloid leukemia treated with imatinib for more than 5 years and approximately half of all first-line treated patients have stable undetectable BCR-ABL using strict sensitivity criteria. Clin Cancer Res. 2007 1;13(23):7080-5.
18. Hughes TP, Saglio G, Kantarjian HM, et al. Early molecular response predicts outcomes in patients with chronic myeloid leukemia in chronic phase treated with frontline nilotinib or imatinib. Blood. 2014 27;123(9):1353-60.
19. Jabbour E, Kantarjian HM, Saglio G, et al. Early response with dasatinib or imatinib in chronic myeloid leukemia: 3-year follow-up from a randomized phase 3 trial (DASISION). Blood. 2014. 23;123(4):494-500
20. Hochhaus A. Educational session: managing chronic myeloid leukemia as a chronic disease. Hematology Am Soc Hematol Educ Program. 2011:128-35.
21. Efficace F, Baccarani M, Breccia M, et al. Health-related quality of life in chronic myeloid leukemia patients receiving long-term therapy with imatinib compared with the general population. Blood. 2011. 27;118(17):4554-60
22. Valent P, Hadzijusufovic E, Schernthaner GH, at al. Vascular safety issues in CML patients treated with BCR/ABL1 kinase inhibitors. Blood. 2015. 5;125(6):901-6.

23. Dulucq, S., Mahon, F. X. Deep molecular responses for treatment‐free remission in chronic myeloid leukemia. Cancer Medicine. 2016. 5(9), 2398-2411.
24. Etienne G, Rea D, Guilhot J et al. Long term follow-up of the French 1 Stop Imatinim Study (STIM1) in chronic myeloid leukemia patients. ASH Annual Meeting Abstract 2015: abs 345.
25. Ross DM, Branford S, Seymour JF, et al. Safety and efficacy of imatinib cessation for CML patients with stable undetectable minimal residual disease: results from the TWISTER study. Blood. 2013 25;122(4):515-22.
26. Mahon F.X., Richter J., Guilhot J. et al. Cessation of Tyrosine Kinase Inhibitors Treatment in Chronic Myeloid Leukemia Patients with Deep Molecular Response: Results of the Euro-Ski Trial. ASH Annual Meeting Abstract 2016: abs 787
27. Hochhaus A. Masszi T. Giles F.J. et al. Treatment-free remission (TFR) in patients (pts) with chronic myeloid leukemia in chronic phase (CML-CP) treated with frontline nilotinib: Results from the ENESTFreedom study. ASCO Annual Meeting 2016: abs 7001
28. Chomel, J. C., Bonnet, M. L., Sorel, N., et al. Leukemic stem cell persistence in chronic myeloid leukemia patients in deep molecular response induced by tyrosine kinase inhibitors and the impact of therapy discontinuation. Oncotarget, 2016. 7(23), 35293-35301.
29. Corbin AS, Agarwal A, Loriaux M, et al. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. J Clin Invest. 2011. 121(1):396-409. Erratum in: J Clin Invest. 2011 Mar 1;121(3):1222.
30. Essers, M.A.G., Trumpp, A. Targeting leukemic stem cells by breaking their dormancy. Molecular oncology, 2010. 4(5), 443-450.
31. Chomel JC1, Turhan AG. Chronic myeloid leukemia stem cells in the era of targeted therapies: resistance, persistence and long-term dormancy. Oncotarget. 2011. 2(9):713-27.
32. Hamilton A, Helgason GV, Schemionek M, et al. Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival. Blood. 2012. 9;119(6):1501-10.
33. Bocchia, M., Ippoliti, M., Gozzetti, A., CD34+/Ph+ cells are still detectable in chronic myeloid leukemia patients with sustained and prolonged complete cytogenetic remission during treatment with imatinib mesylate. Leukemia, 2008 22(2), 426-428.

34. Mustjoki S, Rohon P, Rapakko K, et al. Low or undetectable numbers of Philadelphia chromosome-positive leukemic stem cells (Ph(+)CD34(+)CD38(neg)) in chronic myeloid leukemia patients in complete cytogenetic remission after tyrosine kinase inhibitor therapy. Leukemia. 2010. 24(1):219-22.

35. Defina, M., Ippoliti, M., Gozzetti, A. et al. Evaluation of residual CD34+ Ph+ progenitor cells in chronic myeloid leukemia patients who have complete cytogenetic response during first‐line nilotinib therapy. Cancer, 2012118(21), 5265-5269.

36. Järås M, Johnels P, Hansen N, et al. Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. Proc Natl Acad Sci USA 2010. 107(37):16280-16285.

37. Florian S, Sonneck K, Hauswirth AW, et al. Detection of molecular targets on the surface of CD34+/CD38— stem cells in various myeloid malignancies. Leuk Lymphoma 2006. 47(2):207-222.

38. Herrmann H, Cerny-Reiterer S, Gleixner KV, et al. CD34(+)/CD38(-) stem cells in chronic myeloid leukemia express Siglec-3 (CD33) and are responsive to the CD33-targeting drug gemtuzumab/ozogamicin. Haematologica 2012. 97(2):219-226

39. Dürig J, Rosenthal C, Elmaagacli A, et al. Biological effects of stroma-derived factor-1 alpha on normal and CML CD34+ haemopoietic cells. Leukemia 2000.14(9):1652-1660.

40. Zhang B, Ho YW, Huang Q, et al. Altered microenvironmental regulation of leukemic and normal stem cells in chronic myelogenous leukemia. Cancer Cell 2012. 21(4):577-592.

41. Valent, P., Sadovnik, I., Ráčil, Z., et al. DPPIV (CD26) as a novel stem cell marker in Ph+ chronic myeloid leukaemia. European journal of clinical investigation, 2014. *44*(12), 1239-1245.

42. Herrmann, H., Sadovnik, I., Cerny-Reiterer, S., et al. Dipeptidylpeptidase IV (CD26) defines leukemic stem cells (LSC) in chronic myeloid leukemia. Blood, 2014. 123(25), 3951-3962.

43. Culen M, Borsky M, Nemethova V, et al. Quantitative assessment of the CD26+ leukemic stem cell compartment in chronic myeloid leukemia: patient-subgroups, prognostic impact, and technical aspects. Oncotarget. 2016. 31;7(22):33016-24.