CLINICAL OUTCOME AND BIOLOGICAL CHARACTERISTICS OF CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH NILOTINIB FRONT-LINE

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Contents

Summary .................................................................................................................. 3
1. Background and introduction .................................................................................. 5
  1.1. Chronic Myeloid Leukemia: natural history and treatment results ............... 5
  1.2. Response Monitoring ....................................................................................... 5
  1.3. Nilotinib ........................................................................................................... 7
    1.3.1. Introduction ............................................................................................... 7
    1.3.2. Clinical studies ......................................................................................... 8
    1.3.3. Toxicity profile ........................................................................................ 9
  1.4. Molecular pathways involved in CML ................................................................. 16
  1.5. Leukemic stem cells (Cancer progenitor cells) in CML ........................................ 22
2. Rationale of the study ............................................................................................. 35
3. Methods ................................................................................................................ 38
  3.1. Objective of the trial ......................................................................................... 38
    3.1.1. Primary objective ..................................................................................... 38
    3.1.2. Secondary Objectives .............................................................................. 38
  3.2. Trial Design ..................................................................................................... 38
    3.2.1. Patient selection criteria .......................................................................... 38
    3.2.2. Treatment scheme .................................................................................... 40
    3.2.3. Primary endpoint ...................................................................................... 42
    3.2.4. Secondary endpoints ............................................................................... 42
    3.2.5. Therapeutic regimens, expected toxicity, dose modifications .................. 42
    3.2.6. Clinical evaluation, laboratory tests and follow-up .................................... 44
    3.2.7. Criteria of evaluation ............................................................................... 47
    3.2.8. Statistical methods and data analysis ........................................................ 51
  3.3. Leukemic Stem cell collection and proteomic analysis ........................................ 52
4. Results ................................................................................................................... 54
  4.1. Clinical results .................................................................................................. 54
    4.1.1. Patients characteristics ............................................................................ 54
    4.1.2. Molecular response .................................................................................... 55
    4.1.3. Response according to ELN 2013 recommendations .................................... 58
    4.1.4. Events ....................................................................................................... 59
    4.1.5. Cardiovascular events .............................................................................. 60
    4.1.6. Patient disposition .................................................................................... 60
  4.2. Biological results .............................................................................................. 61
    4.2.1. Samples ..................................................................................................... 61
    4.2.2. Analysis of the phospho-proteomic profile ................................................ 61
    4.2.3 Analysis of the expression of apoptotic proteins .......................................... 63
5. Discussion .............................................................................................................. 66
6. Acknowledgments .................................................................................................. 70
7. References .............................................................................................................. 71
Summary

The present work reports the outcome of the GIMEMA CML WP study CML0811, an independent trial investigating nilotinib as front-line treatment in chronic phase chronic myeloid leukemia (CML). Moreover, the results of the proteomic analysis of the CD34+ cells collected at CML diagnosis, compared to the counterpart from healthy donors, are reported. Nilotinib has been approved as front-line of CML based on the results of the Company-sponsored ENESTnd trial, where nilotinib showed a superior efficacy compared to imatinib. However, cardiovascular thrombotic events have been recently uncovered, rising concerns on the long-term safety profile of nilotinib.

The treatment-free remission is considered one of the most important goals in CML, and a sustained deep molecular response (DMR, MR 4.0 or better) is a pre-requisite for treatment discontinuation. To date, very few data on the stability of the DMR with nilotinib are currently available. Even when treatment is stopped during a stable DMR, about half of the patients eventually relapse due to the persistence of leukemic stem cells resistant to therapy.

Based on these considerations, it is extremely relevant to: a) assess in an independent study the efficacy, including the stability of the molecular response, and the safety, with particular attention to the cardiovascular events, of nilotinib front-line; b) characterize the leukemic stem cells, understanding their differences with the normal counterpart.

One hundred thirty CML patients in early chronic phase have been enrolled in 32 Italian hematologic centers, with a median age at diagnosis of 50 years (range 18-85). After a median follow-up of 24 months all patients are still alive, and only 2 patients progressed to accelerated/blast phase (AP/BP). At the last contact, 107/130 (82%) patients were still on treatment with nilotinib. The main reasons for treatment discontinuation were toxicity in 11 patients (8%), and failures in 5 (4%) patients. Six patients (5%) had a cardiovascular event, including myocardial infarction and arterial thrombosis. Both LDL and HDL cholesterol fractions significantly increased during treatment. The optimal responders at 3, 6, and 12
months according to ELN 2013 recommendations were 82.3%, 80%, and 53%, respectively; failures at the same time points were 10.7%, 3.8%, and 2.3%, respectively. The estimated 24 months cumulative rates of MR 4.0 and MR 4.5 were 47% and 22%, respectively. Overall, 27/57 (47%, or 21% of the total) patients had a sustained MR 4.0; 6/30 (20%, or 5% of the total) patients had a sustained MR 4.5. The estimated 24-month overall-survival, progression-free survival, failure-free survival, and event-free survival were 100%, 98%, 94%, and 73%, respectively.

The proteomic analysis showed that the CD34+ cells from CML patients at diagnosis, compared to a normal counterpart, have: a lower phosphorylation of STAT2, STAT5, and of tyrosine kinases of the Src family (Lck, Fyn, Lyn, and Yes); a lower expression of the catalase, and a higher expression of FADD, HSP60 and HSP70. Taken together, these data show that CML CD34+ cells have a proteomic profile that promotes the quiescence through the inhibition of proliferation, and that favors cell survival despite cytotoxic stimuli. These characteristics may explain, at least partially, why these cells are resistant to the treatment with TKIs, which is highly effective in targeting more differentiated and proliferating cells.

In conclusion, our study confirmed that nilotinib is highly effective in the prevention of the progression to AP/BP, a condition that today is still associated with high mortality rates. Despite the relatively short follow-up, cardiovascular issues, particularly atherosclerotic adverse events (AE), have emerged, and the frequency of these AEs may counterbalance the anti-leukemic efficacy. The deep molecular response rates in our study compare favorably to those obtained with imatinib, in historic cohorts, and confirm the findings of the Company-sponsored ENESTnd study. Considering the increasing rates of deep MR over time, a significant proportion of patients will be candidate to treatment discontinuation in the next years, with higher probability of remaining disease-free in the long term. However, the presence of the additional and complex changes we found at the proteomic level in CML CD34+ cells should be taken into account for the investigation on novel targeted therapies, aimed at the eradication of the disease.
1. Background and introduction

1.1. Chronic Myeloid Leukemia: natural history and treatment results.

Chronic Myeloid Leukemia (CML) is a hematopoietic stem cell disorder associated with a specific chromosomal translocation known as the Philadelphia (Ph) chromosome. The molecular consequence of the translocation is the fusion of the Abl proto-oncogene to the Bcr gene, resulting in the production of a constitutively activated form of the ABL protein-tyrosine kinase, whose activity gives growth advantage to leukemic cells, increases proliferation and cytokine-independent growth, inhibits apoptosis, and alters adhesion pathways. Expression of the BCR-ABL protein is able to induce leukemia in mice.

Clinically, CML progresses through three distinct phases of increasing refractoriness to therapy: chronic phase (CP, median duration without TKI therapy 3 to 4 years), accelerated phase (AP, median survival 8 to 18 months), and blast phase (BP, median survival 3 to 6 months). Most patients present in the chronic phase, which is characterized by splenomegaly and leukocytosis with generally few symptoms.

CML can be effectively treated with imatinib (Glivec, Novartis Pharma): after 6-year follow-up, the International Randomized Study of Interferon vs STI571 (IRIS) showed a cumulative complete cytogenetic response (CCgR) rate of 82%; however, after 8 years, only 56% of the patients were still on imatinib treatment. Moreover, not all patients achieve an optimal response and about 10% of patients of patients lose the previously obtained response, particularly within the first three years of treatment. For these patients second- and third-generations TKIs have been developed: nilotinib and dasatinib have been approved for first and second line treatment; bosutinib, and ponatinib for second line treatment.

1.2. Response Monitoring

Alongside the development of targeted therapies, that allows more patients to achieve optimal responses, the diagnostic procedures have also been refined. The objectives of treatment of CML are the normalization of hematopoiesis
(complete hematologic response - CHR), the elimination of the Ph+ cells from the bone marrow (complete cytogenetic response - CCgR), the reduction of the BCR-ABL transcript levels from samples of peripheral blood by a factor of at least three logs, compared to the standardized baseline (major molecular response - MMR, corresponding to a BCR-ABL/ABL ratio ≤ 0.1% International Scale), and four logs or more (deep molecular response – DMR: MR 4.0 or more)\textsuperscript{12-14}. Assessing the molecular response by quantitatively measuring the BCR-ABL transcripts, using real-time reverse transcriptase polymerase chain reaction (RQ-PCR), is an established parameter of response that can be easily assessed from peripheral blood. Patients achieving a CCgR and a MMR have a better prognosis than patients achieving a CCgR alone\textsuperscript{15}. Standardized molecular monitoring has become widely available in Europe through the efforts of EUTOS cooperation\textsuperscript{16} and, in Italy, through the cooperative efforts of a group of laboratories nationwide which agreed to harmonize their activity and to share results of the molecular monitoring, managed within the frame of the Labnet network. These efforts led to the generation of comparable data on the residual disease.

The response to TKI treatment is the most important prognostic factor. For this reason, the European Leukemia Net, since 2006\textsuperscript{17}, has provided recommendations that define the response to first-line treatment with any TKI. The updated definitions (ELN 2013) are reported in Table 1\textsuperscript{18}.  

Table 1. 2013 ELN definition of response to front-line treatment with TKIs

<table>
<thead>
<tr>
<th></th>
<th>Optimal</th>
<th>Warning</th>
<th>Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>NA</td>
<td>High risk</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Or CCA/Ph+, major route</td>
<td></td>
</tr>
<tr>
<td>3 mo</td>
<td>BCR-ABL1 ≤10%</td>
<td>BCR-ABL1 &gt;10%</td>
<td>Non-CHR</td>
</tr>
<tr>
<td></td>
<td>and/or Ph+ ≤35%</td>
<td>and/or Ph+ 36-95%</td>
<td>and/or Ph+ &gt;95%</td>
</tr>
<tr>
<td>6 mo</td>
<td>BCR-ABL1 &lt;1%</td>
<td>BCR-ABL1 1-10%</td>
<td>BCR-ABL1 &gt;10%</td>
</tr>
<tr>
<td></td>
<td>and/or Ph+ 0</td>
<td>and/or Ph+ 1-35%</td>
<td>and/or Ph+ &gt;35%</td>
</tr>
<tr>
<td>12 mo</td>
<td>BCR-ABL1 ≤0.1%</td>
<td>BCR-ABL1 &gt;0.1-1%</td>
<td>BCR-ABL1 &gt;1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and/or Ph+ &gt;0</td>
<td></td>
</tr>
<tr>
<td>Then, and at any time</td>
<td>BCR-ABL1 ≤0.1%</td>
<td>CCA/Ph− (–7, or 7q−)</td>
<td>Loss of CHR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Loss of CCyR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Confirmed loss of MMR+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mutations</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCA/Ph+</td>
</tr>
</tbody>
</table>

1.3. Nilotinib

1.3.1. Introduction

Nilotinib (Tasigna, AMN107, Novartis Pharma) is an aminopyrimidine derivative, available as an oral formulation: it is a rationally designed second-generation tyrosine kinase inhibitor with improved target specificity over imatinib. The ATP-competitive inhibition of the BCR-ABL protein tyrosine kinase activity prevents the activation of BCR-ABL dependent mitogenic and anti-apoptotic pathways (e.g., PI3 kinase and STAT5). Following the administration to animals, nilotinib is moderately absorbed (approximately 30% bioavailability) and well tolerated. The effects of a systemic exposure (C<sub>max</sub>, AUC) over the range of 50 to 1200 mg once daily have been assessed in patients with imatinib-resistant CML in chronic, accelerated or blast phase, relapsed or refractory Ph+ acute lymphoblastic
leukemia (ALL), or other hematologic malignancies\textsuperscript{19}. With once daily (QD) dosing, the steady-state, the C\textsubscript{max}, and the AUC increased with increasing dose from 50 mg to 400 mg/day in a generally dose-proportional manner, but appeared to plateau at dose levels of 400 mg/day or higher, remaining relatively constant over the dose range from 400 mg to 1200 mg. QD doses of nilotinib 400 mg or 800 mg showed no appreciable differences in serum exposure to drug. Using a twice daily schedule (BID), the dose-limiting exposure has been partially overcome, with a daily steady-state serum nilotinib exposure at a dose of 400 mg BID being approximately 35\% greater than with a dose of 800 mg once daily. However, there was no further relevant increase in nilotinib exposure observed with the administration of the 600 mg BID dose (1200 mg/day). With multiple oral doses of nilotinib, steady-state conditions were achieved by day 8 after initiating nilotinib treatment. The median time to reach C\textsubscript{max} of nilotinib (t\textsubscript{max}) was 3 hours. Drug half-life averaged 17 hours for once daily dosing. CYP3A4 appears to be the major enzyme responsible for the metabolism of nilotinib under in vivo conditions whereas the contribution of CYP2C8 is expected to be minor (~8\%). Nilotinib also seems to inhibit CYP2C8, CYP2C9, CYP2D6, CYP3A4/5, and UGTIA1 activity in clinical settings.

1.3.2. Clinical studies
The efficacy and safety of nilotinib in the treatment of patients who are resistant to or intolerant of imatinib led to its further evaluation in the treatment of newly diagnosed CML\textsuperscript{20-22}. Data from two Investigator-initiated (GIMEMA and MDACC) trials demonstrate high rates of cytogenetic and molecular responses in patients with newly diagnosed CML\textsuperscript{23,24}: the 12-months complete molecular response rates were 7\% in the GIMEMA trial and 10 \% in the MDACC trial. A phase 3 randomized study comparing the efficacy and safety of 300 or 400 mg bid nilotinib with imatinib 400 mg daily in patients with newly diagnosed Ph+ CP-CML (ENESTnd) is actually ongoing\textsuperscript{25}. This study showed a superiority of nilotinib 300 mg or 400 mg BID over imatinib: CCgR rates at 12 months were 80\%, 78\% and 65\%, respectively;
MMR rates at 12 months were 44%, 43% and 22%, respectively. The progression to advanced disease was lower for nilotinib 300 mg bid (2 pts) and nilotinib 400 mg bid (1 pt) compared with imatinib 400 mg (11 pts).

The best cumulative MMR rates at 24 months\(^2\) were 62%, 59% and 37%, for nilotinib 300 mg BID, nilotinib 400 mg BID, and imatinib 400 mg OAD respectively. Recently, the ENESTnd trial has been updated\(^3\): for nilotinib 300 mg BID, nilotinib 400 mg BID, and imatinib 400 mg OAD, MMR rates by 60 months were 77%, 77% and 60% (p < 0.001), respectively; and MR 4.5 rates by 60 months were 54%, 53% and 31% (p < 0.001), respectively. By the 60-month data cut-off date, 17 patients progressed to AP/BC on core treatment, 12 in the imatinib arm, 2 in the nilotinib 300 mg BID arm and 3 in the nilotinib 400 mg BID arm. No additional patients with progression to AP/BC were observed on core treatment in any treatment arm since the 24-month analysis.

Nilotinib 300 mg BID has been shown to be equally effective and better tolerated than 400 mg BID. Novartis Pharma has submitted regulatory applications worldwide, obtaining the registration for the first-line indication in USA, EU and Japan.

1.3.3. Toxicity profile
1.3.3.1. Adverse events
The most frequently reported drug-related adverse events (AEs) in CML-AP and CML-CP patients who are resistant to or intolerant of imatinib are shown in Table 1. At 24 months follow-up, almost all CML patients experienced AEs during the course of the study. In CML-CP patients, the most frequent SAEs were thrombocytopenia (3.4%), neutropenia (2.2%), angina pectoris (2.8%) and pyrexia (2.5%). In CML-AP patients, the most frequent SAEs were thrombocytopenia (8.0%), neutropenia (5.8%), pneumonia (5.1%), and pyrexia (4.4%). The rate of discontinuations due to AEs, regardless of relationship to study medication, was similar for both stages of the disease (19.0% of CML-CP patients and 17.5% of CML-AP patients). For CML-CP, the incidence of AEs leading to discontinuation was low; the most frequent AEs associated with
discontinuation were neutropenia and thrombocytopenia, which occurred in 10 (3.1%) patients each. For CML-AP, the incidence of AEs leading to discontinuation was low; the most frequent AE associated with discontinuation was thrombocytopenia, which occurred in 10 (7.3%) patients.

Table 2. Most frequent drug-related adverse events (more than 5%) at 24-months in CML-AP and -CP patients (Study 2101 E1 and E2)

<table>
<thead>
<tr>
<th>Any event</th>
<th>All grades CML-AP, N=137, n (%)</th>
<th>All grades CML-CP, N=321, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any event</td>
<td>120 (87.6)</td>
<td>304 (94.7)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>52 (38.0)</td>
<td>90 (28.0)</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>31 (22.6)</td>
<td>48 (15.0)</td>
</tr>
<tr>
<td>Rash</td>
<td>29 (21.2)</td>
<td>99 (30.8)</td>
</tr>
<tr>
<td>Anemia</td>
<td>24 (17.5)</td>
<td>42 (13.1)</td>
</tr>
<tr>
<td>Pruritus</td>
<td>24 (17.5)</td>
<td>84 (26.2)</td>
</tr>
<tr>
<td>Lipase increased</td>
<td>18 (13.1)</td>
<td>41 (12.8)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>14 (10.2)</td>
<td>65 (20.2)</td>
</tr>
<tr>
<td>Constipation</td>
<td>13 (9.5)</td>
<td>43 (13.4)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>13 (9.5)</td>
<td>39 (12.1)</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>13 (9.5)</td>
<td>13 (4.0)</td>
</tr>
<tr>
<td>Muscle spasms</td>
<td>13 (9.5)</td>
<td>24 (7.5)</td>
</tr>
<tr>
<td>Nausea</td>
<td>13 (9.5)</td>
<td>79 (24.6)</td>
</tr>
<tr>
<td>Alopecia</td>
<td>12 (8.8)</td>
<td>27 (8.4)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>12 (8.8)</td>
<td>33 (10.3)</td>
</tr>
<tr>
<td>Blood bilirubin increased</td>
<td>11 (8.0)</td>
<td>22 (6.9)</td>
</tr>
<tr>
<td>Headache</td>
<td>11 (8.0)</td>
<td>57 (17.8)</td>
</tr>
<tr>
<td>Hyperbilirubinemia</td>
<td>11 (8.0)</td>
<td>23 (7.2)</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>10 (7.3)</td>
<td>17 (5.3)</td>
</tr>
<tr>
<td>Pyrexia</td>
<td>9 (6.6)</td>
<td>13 (4.0)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>8 (5.8)</td>
<td>23 (7.2)</td>
</tr>
<tr>
<td>Pain in extremity</td>
<td>8 (5.8)</td>
<td>17 (5.3)</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>7 (5.1)</td>
<td>24 (7.5)</td>
</tr>
<tr>
<td>Hypophosphataemia</td>
<td>7 (5.1)</td>
<td>8 (2.5)</td>
</tr>
<tr>
<td>Peripheral edema</td>
<td>7 (5.1)</td>
<td>20 (6.2)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>5 (3.6)</td>
<td>41 (12.8)</td>
</tr>
<tr>
<td>Alanine aminotransferase increase</td>
<td>5 (3.6)</td>
<td>34 (10.6)</td>
</tr>
<tr>
<td>Bone pain</td>
<td>5 (3.6)</td>
<td>24 (7.5)</td>
</tr>
<tr>
<td>Erythema</td>
<td>2 (1.5)</td>
<td>23 (7.2)</td>
</tr>
<tr>
<td>Asthenia</td>
<td>5 (3.6)</td>
<td>21 (6.5)</td>
</tr>
<tr>
<td>Aspartate aminotransferase increase</td>
<td>4 (2.9)</td>
<td>20 (6.2)</td>
</tr>
<tr>
<td>increase</td>
<td>5 (3.6)</td>
<td>20 (6.2)</td>
</tr>
<tr>
<td>Dry skin</td>
<td>0 (0.0)</td>
<td>17 (5.3)</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>3 (2.2)</td>
<td>17 (5.3)</td>
</tr>
</tbody>
</table>

AE = adverse event, AP = accelerated phase, CML = chronic myeloid leukemia, CP = chronic phase, 1The source used for 24-month 2101E1 data (cut-off 29-Aug-2008) and 24-month 2101E2 data (cut-off 20-Apr-2008)
Source: [CAMN107A2101E1] and [CAMN107A2101E2 CSR].
Furthermore, a subgroup analysis evaluated the occurrence of cross-intolerance to nilotinib in imatinib-intolerant patients with CML- CP or AP (n=122; CP:95 AP:27). Only 4/75 (5%) patients with non-hematologic imatinib-intolerance experienced similar grade 3/4 AE on nilotinib. Only 7/40 (18%) patients with hematologic toxicity on imatinib discontinued nilotinib for the same reason (all due to grade 3/4 thrombocytopenia).

The frequency and the degree of non-hematologic AEs in the 5-years update of the first line treatment trial ENESTnd are reported in Table 2.

**Table 3. Most frequently reported study drug-related non-hematologic adverse events (at least 5% in any treatment group) by preferred term (Safety set) - ENESTnd**

<table>
<thead>
<tr>
<th>Preferred term</th>
<th>All grades CTC</th>
<th>grade 3 or 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Imatinib 400 mg QD N=280 n (%)</td>
<td>Nilotinib 300 mg BID N=279 n (%)</td>
</tr>
<tr>
<td>ANY AE</td>
<td>264 (94.3)</td>
<td>257 (92.1)</td>
</tr>
<tr>
<td>Rash</td>
<td>39 (13.9)</td>
<td>93 (33.3)</td>
</tr>
<tr>
<td>ALT increased</td>
<td>17 (6.1)</td>
<td>67 (24.0)</td>
</tr>
<tr>
<td>Headache</td>
<td>29 (10.4)</td>
<td>46 (16.5)</td>
</tr>
<tr>
<td>Nausea</td>
<td>97 (34.6)</td>
<td>39 (14.0)</td>
</tr>
<tr>
<td>Hyper-bilirubinaemia</td>
<td>4 (1.4)</td>
<td>46 (16.5)</td>
</tr>
<tr>
<td>Pruritus</td>
<td>15 (5.4)</td>
<td>49 (17.6)</td>
</tr>
<tr>
<td>AST increased</td>
<td>14 (5.0)</td>
<td>34 (12.2)</td>
</tr>
<tr>
<td>Hypophosphataemia</td>
<td>38 (13.6)</td>
<td>35 (12.5)</td>
</tr>
<tr>
<td>Alopecia</td>
<td>16 (5.7)</td>
<td>29 (10.4)</td>
</tr>
<tr>
<td>Dry skin</td>
<td>13 (4.6)</td>
<td>27 (9.7)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>35 (12.5)</td>
<td>29 (10.4)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>37 (13.2)</td>
<td>34 (12.2)</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>23 (8.2)</td>
<td>23 (8.2)</td>
</tr>
<tr>
<td>Lipase increased</td>
<td>10 (3.6)</td>
<td>30 (10.8)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>52 (18.6)</td>
<td>16 (5.7)</td>
</tr>
</tbody>
</table>
1.3.3.2. Lipid and glucose elevations

Lipid and glucose elevation represent important adverse events that have been associated with nilotinib treatment, and with potential important implications on the overall cardiovascular risk. In the ENESTnd Study\textsuperscript{25}, considering all the observed adverse events (not only drug-related events) the proportion of patients with high cholesterol (almost all grade 1 or 2) was greater in the nilotinib treated patients compared to the imatinib ones (27.6% and 26.7% in the nilotinib 300 mg BID and 400 mg BID groups, versus 3.9% in the imatinib group). Also the proportion of patients with post-baseline LDL values >100 mg/dL was higher in the nilotinib 400 mg BID group (43.3%) and the nilotinib 300 mg BID group (38.4%) as compared to imatinib group (18.6%). Triglyceride abnormalities were slightly more frequent in the nilotinib treated patients compared to the imatinib ones (11.8% and 10.5% in the nilotinib 300 mg and 400 mg groups, vs. 7.9% in the imatinib group).

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Group 1 (n, %)</th>
<th>Group 2 (n, %)</th>
<th>Group 3 (n, %)</th>
<th>Group 4 (n, %)</th>
<th>Group 5 (n, %)</th>
<th>Group 6 (n, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal pain</td>
<td>22 (7.9)</td>
<td>28 (10.0)</td>
<td>24 (8.7)</td>
<td>2 (0.7)</td>
<td>3 (1.1)</td>
<td>0</td>
</tr>
<tr>
<td>Muscle spasms</td>
<td>83 (29.6)</td>
<td>25 (9.0)</td>
<td>24 (8.7)</td>
<td>3 (1.1)</td>
<td>0</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>86 (30.7)</td>
<td>24 (8.6)</td>
<td>20 (7.2)</td>
<td>9 (3.2)</td>
<td>1 (0.4)</td>
<td>0</td>
</tr>
<tr>
<td>Amylase increased</td>
<td>9 (3.2)</td>
<td>18 (6.5)</td>
<td>19 (6.9)</td>
<td>3 (1.1)</td>
<td>2 (0.7)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>Constipation</td>
<td>7 (2.5)</td>
<td>27 (9.7)</td>
<td>19 (6.9)</td>
<td>0</td>
<td>0</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Oedema peripheral</td>
<td>49 (17.5)</td>
<td>15 (5.4)</td>
<td>19 (6.9)</td>
<td>0</td>
<td>1 (0.4)</td>
<td>0</td>
</tr>
<tr>
<td>Erythema</td>
<td>9 (3.2)</td>
<td>7 (2.5)</td>
<td>17 (6.1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>11 (3.9)</td>
<td>17 (6.1)</td>
<td>16 (5.8)</td>
<td>0</td>
<td>0</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>0</td>
<td>9 (3.2)</td>
<td>16 (5.8)</td>
<td>0</td>
<td>1 (0.4)</td>
<td>0</td>
</tr>
<tr>
<td>Asthenia</td>
<td>24 (8.6)</td>
<td>26 (9.3)</td>
<td>15 (5.4)</td>
<td>0</td>
<td>1 (0.4)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>16 (5.7)</td>
<td>13 (4.7)</td>
<td>15 (5.4)</td>
<td>0</td>
<td>0</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Hyperglycaemia</td>
<td>2 (0.7)</td>
<td>12 (4.3)</td>
<td>15 (5.4)</td>
<td>0</td>
<td>2 (0.7)</td>
<td>4 (1.4)</td>
</tr>
<tr>
<td>Blood ALP increased</td>
<td>7 (2.5)</td>
<td>6 (2.2)</td>
<td>14 (5.1)</td>
<td>1 (0.4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bone pain</td>
<td>10 (3.6)</td>
<td>11 (3.9)</td>
<td>14 (5.1)</td>
<td>1 (0.4)</td>
<td>0</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Pain in extremity</td>
<td>23 (8.2)</td>
<td>13 (4.7)</td>
<td>9 (3.2)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>Face oedema</td>
<td>37 (13.2)</td>
<td>1 (0.4)</td>
<td>6 (2.2)</td>
<td>1 (0.4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Eyelid oedema</td>
<td>50 (17.9)</td>
<td>2 (0.7)</td>
<td>5 (1.8)</td>
<td>1 (0.4)</td>
<td>0</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Weight increased</td>
<td>18 (6.4)</td>
<td>8 (2.9)</td>
<td>4 (1.4)</td>
<td>2 (0.7)</td>
<td>3 (1.1)</td>
<td>0</td>
</tr>
<tr>
<td>Periorbital oedema</td>
<td>40 (14.3)</td>
<td>1 (0.4)</td>
<td>3 (1.1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Elevated glucose values were more frequent in the nilotinib treated patients than in the imatinib ones. The proportion of patients with grade 3/4 increased serum glucose was higher in the nilotinib groups (7.2%; 20 patients in the nilotinib 300 mg BID and 6.9%; 19 patients in the nilotinib 400 mg BID), while grade 3/4 glucose increase was reported only in one patient (0.4%) in the imatinib group.

The proportion of patients with glycosylated hemoglobin ≥5.7% at any time in the study was higher in the nilotinib 400 mg BID arm (24.2%) and nilotinib 300 mg BID arm (21.9%) as compared to the imatinib arm (18.2%). Also, the proportion of patients with glycosylated hemoglobin ≥6.5% was higher in the nilotinib 300 mg BID group (11.8%) and the nilotinib 400 mg BID group (11.6%) as compared to imatinib group (3.6%).

1.3.3.3. Ischemic vascular or ischemic cardiovascular events

Peripheral ischemic vascular adverse events have been reported with unusual incidence in nilotinib treated patients\textsuperscript{28,29}; the underlying mechanisms are object of active investigations.

In the ENESTnd Study, newly diagnosed or worsened Ischemic Vascular and Ischemic Cardiovascular Events such as Ischemic Heart Disease (IHD), Ischemic Cerebrovascular Event (ICVE) or Peripheral Artery Occlusive Disease (PAOD) have occurred in a relatively small number of CML-CP patients treated with nilotinib. However, such events have been reported with higher frequency in the nilotinib treatment arms compared with the imatinib treatment arm.

Up to the data cut-off for the 60 Month analysis (30-Sep-2013)\textsuperscript{27}, the number of patients reported with events is as follows:

- Nilotinib 300mg BID: IHD, 11 (3.9%); ICVE, 4 (1.4%); PAOD, 7 (2.5%)
- Nilotinib 400mg BID: IHD, 24 (8.7%); ICVE, 9 (3.2%); PAOD, 7 (2.5%)
- Imatinib 400mg QD: IHD, 5 (1.8%); ICVE, 1 (0.4%); PAOD, 0 (0.0%)

The majority of reported ischemic vascular and ischemic cardiovascular events were in patients with associated risks factors (e.g., advanced age, hypertension, hyperlipidemia, hypercholesterolemia, smoking, diabetes mellitus, pre-existing
Peripheral vascular disease). The background incidence of these events has not been established for the CML patient population. However, other studies reported an increased incidence of vascular events in nilotinib treated patients compared to imatinib ones.\textsuperscript{30,31}

**Ischemic heart disease**

Ischemic heart disease (IHD) group included angina pectoris, coronary artery disease, acute myocardial infarction, coronary artery stenosis, myocardial infarction, myocardial ischaemia, angina unstable, blood creatin-phosphokinase MB increased, coronary artery occlusion, coronary artery restenosis, and troponin increased. AEs grouped under IHD were more frequent with nilotinib than with imatinib: there were 11 patients (3.9\%) in the nilotinib 300 mg BID group and 24 patients (8.7\%) in the nilotinib 400 mg BID group with IHD, compared to five patients (1.8\%) in the imatinib group. Fourteen patients in the nilotinib 400 mg BID group and one patient in the nilotinib 300 mg BID group had AEs grouped under IHD that were considered study drug-related. Most of the IHD events were considered serious and were grade 3/4. Ten patients (3.6\%) in the nilotinib 400 mg BID group discontinued study drug due to the events grouped under ischemic heart disease. The cumulative incidence of such events continue to increase over time: since the 48-month data cut-off, there were 12 additional patients with IHD; two in the imatinib group with no suspected relationship to study treatment, and ten in the nilotinib 400 mg BID group, and five were considered nilotinib-related.

**Peripheral arterial occlusive disease**

Peripheral arterial occlusive disease (PAOD) group included peripheral arterial occlusive disease, peripheral artery stenosis, intermittent claudication, peripheral ischemia, and arterial occlusive disease.

Since the beginning of the study there were 14 patients with reported PAOD: no patients in the imatinib group, seven patients (2.5\%) each in the nilotinib 300 mg BID and 400 mg BID group. Serious PAOD events were reported for nine patients (four in the nilotinib 300 mg BID group and five patients in the nilotinib
400 mg BID group). Seven patients (three in nilotinib 300 mg BID group and four in nilotinib 400 mg BID group) had study drug related peripheral arterial occlusive disease. One patient in nilotinib 300 mg BID group discontinued the study treatment due to PAOD and three patients in nilotinib 400 mg BID group discontinued the study drug due to peripheral artery stenosis and intermittent claudication. Since the 48-month data cut-off, there were five patients (three in the nilotinib 300 mg BID group and two in the nilotinib 400 mg BID group) with newly reported PAOD.

Ischemic cerebrovascular events
Ischemic cerebrovascular events group included cerebrovascular accident, ischemic stroke, transient ischemic attack, carotid artery stenosis, cerebral infarction, cerebral ischemia, amaurosis fugax, basilar artery stenosis, and cerebrovascular disorder.

At the 60-month data cut-off, 14 patients were reported with AEs grouped under ischemic cerebrovascular events: one patient (0.4%) in the imatinib group, four patients (1.4%) in the nilotinib 300 mg BID group and nine patients (3.2%) in the nilotinib 400 mg BID group. The majority of patients (11 patients) were reported with AEs that were considered serious by investigators (one patient in imatinib group, four patients in nilotinib 300 mg BID group and six patients in nilotinib 400 mg BID). Since the 48-month data cut-off, there were five additional patients with reported ischemic cerebrovascular events (one in the nilotinib 300 mg BID group and four in the nilotinib 400 mg BID group).
1.4. Molecular pathways involved in CML

Several molecular events are implicated in the disease, with BCR-ABL possessing the capacity to target a number of intermediary adapter molecules (including, GRB2, CBL and CRKL) that have been shown to be important in the activation of multiple signal transduction cascades facilitating unregulated proliferation and inappropriate survival of malignant cells\textsuperscript{32}. Specifically, results from co-immunoprecipitation experiments observed the formation of stable complexes between BCR-ABL and several adapter proteins such as CRKL, SHC, CBL, p62DOC, and PI3-kinase\textsuperscript{33,34}. In addition, tyrosine phosphorylation of BCR-ABL at specific residues regulated the binding of other proteins such as GRB2\textsuperscript{35}. As a result, important pro-survival, proliferative and anti-apoptotic pathways are activated including RAS, STAT5, and Akt. Conversely, functional cooperation between these pathways is required to promote the full oncogenic activity of BCR-ABL\textsuperscript{36}.

**Signal transducer and activator of transcription 5 (STAT5).** The importance of the transcription factor STAT5 in chronic myeloid leukemia is well established. Over-expression of a constitutively active STAT5 protein in total bone marrow and long-term hematopoietic stem cells results in the induction of a CML-like condition comparable to the BCR-ABL induced disease\textsuperscript{37}. Conversely, deletion of the Stat-5 gene locus prevented the development of myeloid or lymphoid leukemia in primary murine recipients of bone marrow transduced with a retrovirus encoding BCR-ABL\textsuperscript{38}. Overall, STAT5 has been shown to have the capacity to mediate anti-apoptotic mechanisms and enhance cell viability via the up-regulation of genes such as BCL-XL and MCL1, in addition to stimulating cell proliferation\textsuperscript{39}. The BCR-ABL oncoprotein is required in order to phosphorylate a critical tyrosine residue that regulates oligomerization of STAT5, and its subsequent nuclear translocation and DNA binding\textsuperscript{40,41}. Interestingly, STAT5 has been implicated in the development of drug resistance to tyrosine kinase inhibitors (TKI; first-line CML therapy). Enhanced STAT5 expression has shown
to result in the increased probability of acquiring BCR-ABL mutations, as well as contributing to a loss of responsiveness in prolonged disease\textsuperscript{42}. Low expression levels of STAT5 correlate with increased sensitivity to treatment \textit{in vitro}, while enhanced expression leads to a decreased therapeutic response\textsuperscript{43}. Warsch and co-workers reported a highly significant association between the expression level of STAT5 mRNA and the occurrence of BCR-ABL mutations in a cohort of fifty CML patients, with the production of reactive oxygen species (ROS) mediating the effect\textsuperscript{42}. The upregulation of STAT5 also has been shown to increase the number and rate of double-stranded breaks contributing to the enhanced mutation rate.

\textbf{Janus kinase 2 (Jak2).} Evidence suggests that the STAT5/Jak pathway is involved in the signalling of more than fifty growth factors and cytokines, regulating various cellular events including proliferation, differentiation, apoptosis, survival, and migration\textsuperscript{44,45}. In physiological conditions, Jak2 possesses a functional role in immune cell development and hematopoiesis. Activation of Jak2 may also be observed in the initiation and maintenance of cancer, with the discovery of a single point mutation within the non-receptor tyrosine kinase Jak2 (a substitution of a valine residue by phenylalanine at amino acid 617, resulting in Jak2V617F) responsible for facilitating the development of a subset of myeloproliferative disorders\textsuperscript{46}. In addition, as the primary activator of STAT5, it is hypothesized that Jak2 is an essential component of BCR-ABL-driven leukemogenesis\textsuperscript{40}.

Activation of Jak2 via BCR-ABL phosphorylation, has been verified in several cell lines expressing different BCR-ABL variants, as well as in leukemic cells derived from CML patients\textsuperscript{47}. Although inhibition of its kinase activity has been reported to induce apoptosis in BCR-ABL expressing leukemia cells, recent studies suggest that complete deletion of the \textit{Jak2} gene significantly accelerate disease development with increased white blood cell counts and severe splenomegaly\textsuperscript{48}. It has been proposed that as an important component of BCR-ABL signaling, Jak2 is important in the regulation of Ph+ cells through the capacity to mediate
disease maintenance and progression\textsuperscript{49,50}. Due to the conflicting literature however, further study is required in order to completely elucidate the role of Jak2 in CML as well as confirm its validity as a therapeutic target.

**Mitogen-activated protein kinase (MAPK) pathway.** The MAPK pathway is involved in the regulation of various cellular processes, including proliferation, differentiation, and survival\textsuperscript{51}. Activation of MAPK signalling via Ras results in the nuclear translocation of extracellular signal regulated kinase (ERK) in order to effect gene expression. BCR-ABL has been determined to be a potent activator of Ras, with autophosphorylation of tyrosine 177 within the BCR first exon providing a docking site for the adapter molecule Grb-2, and subsequent stabilization of Ras in its active GTP-bound form\textsuperscript{35}. Interestingly, activating mutations in Ras are rarely observed in Ph+ CML suggesting that BCR-ABL is required for the constitutive activation of Ras and therefore, the regulation of downstream mechanisms\textsuperscript{52}. Mitogen-activated protein kinase kinase (MEK) is important in the phosphorylation of ERK, promoting cell proliferation\textsuperscript{53}. Recently, the importance of a persistently high level of MEK-dependent negative feedback was published, with the ability to facilitate BCR-ABL-mediated oncogene addiction by regulating myeloid growth factor receptor (GF-R) signaling\textsuperscript{54}. In addition, the anti-apoptotic factor Mcl-1 is also constitutively expressed via Ras activation in BCR-ABL-expressing cells enhancing survival\textsuperscript{55}. A number of MAPK pathways have been implicated in the pathogenesis of Ph+ disease contributing to malignant transformation, including the main MAPK family members: ERK1/2, ERK5, c-Jun N-terminal kinase (JNK) and p38 MAPK. Extensive research involved in the determination of the cellular pathways involved in the pathogenesis of BCR-ABL+ CML has been conducted, with the activation of the ERK1/2 pathway important in disease development and progression. In embryonic stem cells transformed by BCR-ABL, ERK1/2 is constitutively activated\textsuperscript{56}. Conversely, the oncogenic potential of BCR-ABL is enhanced by Ras via the activation of the ERK signaling cascade\textsuperscript{57}. Important in cellular survival, activation of the ERK1/2 pathway is also thought to be important in the down-
regulation of the pro-apoptotic factor Bcl-2 interacting mediator of cell death (BIM) in both hematopoietic progenitor cells and BCR-ABL transformed murine bone marrow-derived Ba/F3 cells\textsuperscript{55}. Furthermore, the ERK1/2 pathway has been implicated as a mediator in the BCR-ABL-induced activation of the transcription factor STAT3 in both primary CML progenitor cells and in murine embryonic stem cells\textsuperscript{58}. Evidence suggests that ERK1/2 may also regulate BCR-ABL-mediated disruption of the translational-regulator heterogenous nuclear ribonucleoprotein K (hnRNP-K) which has been shown to have an important role in the development of blast phase CML\textsuperscript{59}. Despite this, inhibition of ERK1/2 signaling alone failed to significantly induce cell death in BCR-ABL+ leukemic cells, indicating that these cells utilize multiple survival pathways\textsuperscript{60}. Interestingly, a number of studies have implicated a paradoxical activation of the ERK1/2 pathway in TKI resistance\textsuperscript{61}. In the K562 cell line resistant to TKI treatment, imatinib effectively attenuated the phosphorylation of all MAPK signaling, except ERK1/2\textsuperscript{62}. Additionally, co-incubation with an ERK1/2 inhibitor (U0126) resulted in reduced phospho-ERK1/2 levels in resistant K562 cells, restoring sensitivity to TKI therapy. It thus proposed that activation of ERK1/2 may be independent of BCR-ABL in select cases of CML.

Observed as a parallel MAPK pathway to ERK1/2, ERK5 has been recognized as a component of regulatory cell signaling, with the capacity to mediate the transcription of myocyte-specific enhancer factor 2C (MEF2C), an important factor in the differentiation of myeloid cells, influencing the cellular fate between monocyte and granulocyte\textsuperscript{63}. Buschbeck and co-workers investigated the role of ERK5 in pro-survival signaling of BCR-ABL+ leukemia cells\textsuperscript{64}. Results indicated that BCR-ABL tyrosine kinase activity may affect the ERK5 pathway by at least two independent mechanisms: (1) through the capacity to mediate ERK5 activation and, (2) by increasing the protein level of ERK5. Subsequent experiments aimed to provide insight regarding the functional association between ERK5 and oncogenic BCR-ABL signaling, with the basal activity of ERK5 enhancing cellular transformation of Rat-1 fibroblasts and contributing to the cellular survival of the human megakaryoblastic leukemia cell line, MEG-01.
Suppression of the p38 MAPK signaling pathway appears to contribute to BCR-ABL leukemogenesis via the ability to mediate growth inhibitory and apoptotic mechanism in BCR-ABL+ cells\textsuperscript{65}. Ectopic expression of BCR-ABL in embryonic stem cells was shown to attenuate p38 MAPK activation. Furthermore, studies indicate that BCR-ABL-mediated leukemic transformation may progress in part by nuclear factor κB (NFκB) activation via the p38 MAPK pathway\textsuperscript{66}. Studies have shown that imatinib may activate p38 MAPK and its downstream molecules, MapKapK2 and MSK1 in primary leukemic progenitor cells isolated from CML patients\textsuperscript{67}. Recently, it was shown that the phosphorylation of histone 2A family, member X (H2AX) is regulated by p38 MAPK and required for the induction of apoptosis in K562 cells\textsuperscript{68}. In contrast, Galan-Moya and co-workers reported that constitutive active ABL may activate p38 MAPK in an independent mechanism to its innate tyrosine kinase activity and, via its ability to stabilize M KK6, an upstream activator of p38 MAPK\textsuperscript{69}. Elucidation of the mechanisms involved in the interactions between BCR-ABL and p38 MAPK is thus important in order to assess its role in disease progression, and therapeutic intervention.

**Phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway.** The PI3K/Akt pathway is well characterized in CML, with its constitutive activation in BCR-ABL+ cells observed to possess an important role in cell survival and malignant transformation dependent on the BCR-ABL oncogene\textsuperscript{70,71}. Subsequent phosphorylation of critical survival proteins by Akt including transcription factor regulator glycogen synthase kinase 3β (GSK3β), is important in regulating downstream events resulting in a decrease in the susceptibility of cells to undergo apoptosis\textsuperscript{72}. Naughton and co-workers reported that elevated ROS production (expression of BCR-ABL is associated with increased production of intracellular ROS) in cells expressing BCR-ABL was found to activate the PI3K/Akt pathway through the ability to regulate important factors and downstream effectors including Akt, GSK3β, β-catenin, and Mcl-1\textsuperscript{70}. The tumour suppressor gene, phosphatase and tensin homologue deleted on chromosome-10 (PTEN) has also been shown to be down-regulated by BCR-ABL in CML.
progenitor cells, with the deletion accelerating disease development through the regulation of the \( Akt1 \) gene\(^{73}\). The anti-apoptotic signaling of PI3K/Akt has also been shown to contribute to TKI resistance, with the inhibition of PI3K enhancing the efficacy of imatinib\(^ {74} \). Demonstration of imatinib treatment activating the PI3K/Akt pathway in BCR-ABL+ cells and primary leukemia cells \textit{in vitro}, as well as in a chronic phase CML patient \textit{in vivo} was reported\(^ {75} \). It was found that this activation was critically important in mediating survival during the early phases of resistance acquisition, and was prior to the manifestation of BCR-ABL-dependent resistance mechanisms. Similarly, in five Ph+ cell lines observed to be TKI-resistant despite no underlying mutations or other molecular aberrations indicated in conventional resistance, the activation of the PI3K/Akt pathway was unaffected following imatinib treatment\(^ {76} \). Inhibition of Akt1 however led to apoptosis in these cell lines, indicating that select forms of TKI resistance are attributable to mechanisms independent of BCR-ABL, including the constitutive activation of PI3K/Akt signaling. Most recently, the drug efflux pump, ATP-binding cassette sub-family G member 2 (ABCG2) implicated in multidrug resistance (MDR), and observed to be upregulated in TKI-resistant K562 cell lines, was involved in MDR in CML through the PI3K/Akt signaling pathway, and more specifically via PTEN down-regulation\(^ {77} \). More specifically, ABCG2 function was correlated with drug resistance and disease progression, mediated at least in part, by Akt activation.

\textbf{Focal adhesion signaling pathway.} Expression of the BCR-ABL oncoprotein has been demonstrated to affect hematopoietic cell adhesion to the bone marrow via a number of mechanisms, including the ability to mediate important interactions between cellular adhesion receptors and protein components of the extracellular matrix (ECM)\(^ {78} \).
1.5. Leukemic stem cells (Cancer progenitor cells) in CML

Due to the finding that TKI therapy may only induce cell death in proliferating mature cells, coupled with the increasing biological evidence for a population of malignant cells with self-renewal properties that may initiate cancer development, it is suggested that a reservoir of primitive quiescent progenitor cells insensitive to TKI treatment is present in residual disease and is responsible for CML relapse and progression\textsuperscript{79,80}. Interestingly, incubation of CML stem cells with TKIs \textit{in vitro} led to an increased number of quiescent progenitor cells compared to untreated cells\textsuperscript{80-82}. As a rare phenotypically distinct subpopulation of cancer cells that are thought to be responsible for tumorigenesis, it is proposed that an anticancer therapeutic strategy that targets CML stem-like cells may effectively eradicate the disease with a decreased risk of reoccurrence and phase advancement.

1.5.1. Biological identification and characterization of CML progenitor cells

The characterization of CML progenitor cells has been difficult with a selective method for their isolation not yet established, however a focus on subsets of normal and leukemic human hematopoietic (CD34+) cells is of particular interest in research to date. The CD34+CD38- /Lin- fraction of the leukemic clone has been implicated as an enriched subset which gives rise to such CML stem-like cells\textsuperscript{83,84}. They may also be further refined by their aldehyde dehydrogenase (ALDH) 1 activity, with high ALDH expression in CML stem-like cells capable of engrafting mice compared to more differentiated CML progenitor cells within the CD34+/CD38- population\textsuperscript{85}. However, normal hematopoietic stem cells may also exhibit this phenotype, which demonstrates a requirement for the identification of additional markers that may distinguish CML progenitor cells from the normal population\textsuperscript{86}. A number of markers have been described as differentially expressed on CML progenitor cells in comparison to normal bone marrow stem cells.
Genome-wide profiling of CML stem-like cell populations via microarray analysis resulted in the finding that CML subsets display greater variability in the gene expression patterns than their normal counterparts. Thirty-one transcripts were found to be upregulated in CML CD34+/CD38-ALDHhigh cells in comparison to normal CD34+/CD38-ALDHhigh or CD34+/CD38- cells, including genes in key cell signaling and metabolic pathways.

Functional annotation of the genes highlighted several plasma membrane-associated genes, with DPP-IV (CD26), IL-2RA (CD25), RAB31, PTPRD, CACNA1D, IL-1RAP, SLC4A4, and KCNK5 all upregulated and exhibiting cell surface protein localization. Microarray expression levels were further verified by qRT-PCR. Expressed on normal myeloid cells as well as on CD34+ blast cells in acute myeloid leukemia, the cell surface antigen CD33, has also been shown to be expressed at significantly higher levels on stem cell-enriched CD34+/CD38-leukemic cells compared to normal CD34+/CD38- stem cells.

Samples collected from chronic phase CML patients, CD33 was found to be expressed invariably in the majority of cells, in comparison to the highly variable levels observed in those stem-like cells from patients with accelerated or blast phase disease. The surface antigen CD44 has also been found to be upregulated on CML BCR-ABL-expressing leukemic stem-like cells from human bone marrow. Similar results were also observed from bone marrow samples of mice with CML-like leukemia, with decreased CD44 levels observed in malignant progenitors from the blood or spleen, suggested that CD44 may be involved in the retention of BCR-ABL+ stem cells in the bone marrow stroma. The identification of the IL-1 receptor accessory protein (IL-1RAP) as a candidate marker of CML has also been widely reported.

The adenosine A1 receptor has also been implicated, with differential mRNA expression and protein levels observed on CD34+ CML cells compared to their normal counterparts.

A recent study from Nievergall and co-workers showed that CD123 expression was increased in CD34+/CD38- of both chronic-phase and blast-phase CML patients. In addition, IL-3 plasma levels in chronic-phase CML patients was
seen to be higher at diagnosis relative to plasma samples of healthy donors, and reduced in matched samples of patients treated with TKIs.  

Various studies have also displayed an importance of CD117 (c-Kit) expression in the identification of CD34+ CML progenitor cells\(^93-95\). CD117 is a transmembrane receptor with tyrosine kinase activity and is widely expressed in CD34+ cells including hematopoietic stem cells and myeloid progenitor cells for their physiological development and survival. The proportion of CD34+/CD117+ cells is found to be higher in patients with CML in comparison to healthy subjects. In CML, it is suggested that the upregulation of CD117 on CD34+ progenitor cells is critical in the activation of survival mechanisms independent of BCR-ABL.

CD25, the α-chain of the IL-2 receptor (IL-2Rα), is a subunit of the high-affinity IL-2 receptor and is involved in the transduction of the cytokine to regulate cell survival and proliferation. Using a CML-like mouse model of myeloproliferative disease, it was demonstrated that CML progenitor cells may be divided into CD25+/Lin-/Sca-1+/c-Kit+ (LSK) cells and CD25- LSK cells\(^96\). Similar results were reported in a study, with CD25 aberrantly and specifically expressed on human CD34+/CD38-Lin- CML progenitor cells in comparison to the finding that CD25 was undetectable or weakly expressed on normal hematopoietic stem cells.

Most recently, the surface enzyme CD26 (DPP-IV) was identified as a specific, reliable, and functionally important marker of leukemic stem cells in CML\(^97\). Results from gene array, PCR and flow cytometry studies found that CD26 was invariably expressed on progenitor cells in all CML patient bone marrow, but failed to be detected on CD34+/CD38- stem cells in normal samples or on cancer stem-like cells in other myeloid malignancies. Furthermore, CD26 was not detected on more mature CD34+/CD38+ progenitor cells in CML patients. An association between the expression of CD25 and CD26 on CML stem-like cells was also observed, with only CD26+ cells found to express the BCR-ABL1 gene by FISH and qPCR. Notably, the expression of CD26 was not dependent on BCR-ABL1, or inhibited by imatinib treatment. Further study is required in order to assess the potential of CD26 as a therapeutic target, although preliminary data
1.5.2. Molecular pathways involved in the survival and function of CML leukemic stem cells

Despite the biological evidence for a CML stem cell population that possesses the capacity to sustain disease, the immunophenotypic identification of a leukemic stem-like cell remains to be completely elucidated. In fact, CML progenitor cells (CD34+ cells) are thought to be similar to normal hematopoietic stem cells with self-renewal properties as well as the ability to give rise to a heterogeneous population of cells, and only differ by the presence of the bcr-abl gene mutation. Interestingly however, is the finding that these CML progenitor cells are not dependent on the BCR-ABL oncoprotein for survival. This is perhaps the most important consideration to understanding the factors that contribute to the survival of CML stem-like cells and their innate resistance to TKI therapy. Therefore, complete elucidation of the mechanisms involved in the functional maintenance and survival of CML progenitor cells is required in order to determine molecular pathways alternative to BCR-ABL which may be therapeutically targeted, ultimately leading to their eradication and/or sensitization to TKI treatment.

ALOX5 and lipid metabolism. Important in a number of processes including oxidative stress response, lipid metabolism, and synthesis of leukotriene B4, arachidonate 5-lipoxygenase (ALOX5) has been shown to be upregulated by BCR-ABL in CML stem-like cells. In addition, administration of TKI failed to affect the increase in both gene expression and function of ALOX5, suggesting that this upregulation was independent of kinase activity. Chen and co-workers further demonstrated that ALOX5 is critical in disease development, with Alox5-null mice failing to develop CML following implantation of BCR-ABL-transduced bone marrow cells. Co-expression of BCR-ABL and Alox5 in mice resulted in disease induction, confirming the finding. More specifically, ALOX5 was observed...
to regulate the function of CML stem-like cells exclusively, with a deficiency leading to a reduction in the bone marrow affecting differentiation, cell division and survival of the CML progenitor cells, while maintaining the function of normal hematopoietic stem cells. Furthermore, comparative DNA microarray analysis was performed in order to identify the pathways regulated by the Alox5 gene in CML stem-like cells implicated the macrophage scavenger receptor (Msr1) as a candidate gene, which has been shown to be down-regulated via BCR-ABL and restored by an Alox5 deficiency in cells\textsuperscript{100}. The loss of functional Msr1 was shown to accelerate CML development in murine recipients of BCR-ABL-transduced bone marrow cells, as well as enhance CML progenitor cell function by inhibiting apoptosis and regulating the cell cycle to increase proliferation. Inhibition of ALOX5 function via the selective 5-lipoxygenase (5-LO) inhibitor Zileuton, has been demonstrated in a CML murine model\textsuperscript{99} with promising results. Due to these findings, clinical trials were initiated in order to evaluate the safety of Zileuton in combination with TKIs in CML patients (clinicaltrials.gov). A phase I study of the 5-LO inhibitor with imatinib was terminated with no results published (NCT01130688), and another involving its combination with dasatinib is currently recruiting eligible patients (NCT02047149).

**Autophagy.** The term autophagy is derived from the Greek words “auto” (self) and “phagy” (eating) and was firstly used to describe intracellular vesicles that contained degraded cytoplasmic material. Since then, autophagy has been characterized as a cell survival pathway that functions to degrade and recycle cellular components, such as aged proteins and organelles, that can be re-used to generate ATP and essential building blocks during nutrient and/or oxygen deprivation to maintain homeostasis\textsuperscript{101}.

Autophagy has been shown to be induced by various drugs in CML cells, including TKI treatment in both CML cell lines and primary stem/progenitor (CD34+CD38-) cells\textsuperscript{102}. Specific autophagy inhibition, either with ATG7 or ATG5 knockdown, or pharmacological inhibition using cloroquine (CQ), resulted in enhanced TKI-induced death in CML cell lines and primary CML stem cells.
These promising results have led to the initiation of clinical trials, where hidroxy-CQ is being tested in combination with imatinib in imatinib-sensitive patients\textsuperscript{103}. The fact that autophagy is induced following BCR-ABL inhibition suggests that BCR-ABL down-regulates autophagy. In support of this hypothesis, Altman et al.,\textsuperscript{104} demonstrated that BCR-ABL expressing cells exhibited low basal autophagy, but were highly dependent on it. They also showed that autophagy inhibition following Atg3 deletion in BCR-ABL expressing cells led to increased p53 phosphorylation and accumulation, as well as increased expression of the p53 target gene, p21 and the pro-apoptotic Bcl-2 family protein Puma. However, it was not clear if autophagy inhibition had any effect on mitochondrial number or function that could possibly lead to increased DNA damage, as has been shown in normal HSCs\textsuperscript{105}.

The exact mechanism by which BCR-ABL suppresses autophagy is not entirely clear. However, two recent papers have shed some light on this. Firstly, Sheng et al., showed that BCR-ABL, through the PI3K/Akt pathway, transcriptionally up-regulated activating trans-cripton factor 5 (ATF5) in a FoxO4 dependent manner\textsuperscript{106} and ATF5 in turn, stimulated mTORC1 transcription, required for autophagy inhibition. So this model suggests that BCR-ABL not only activates mTORC1 kinase activity, but also leads to increased mTORC1 transcription. The authors further showed that imatinib-induced autophagy was dependent on inhibition of the PI3K/Akt/mTORC1 pathway, as ectopic expression of constitutively active PI3K (PI3KCAE545K) suppressed autophagy induced by imatinib. Secondly, Yu et al., showed that imatinib inhibited expression of microRNA-30a (mir-30a) in CML cells leading to autophagy induction and up-regulation of BECLIN1 and ATG5 expression\textsuperscript{107}. Taken together this suggests that BCR-ABL expression inhibits autophagy by at least two mechanisms 1) in an mTORC1 independent manner by inducing mir-30a expression and 2) in an mTORC1 dependent manner by inducing mTORC1 activity/expression. To support the involvement of active mTORC1 in autophagy inhibition in CML cells, OSI-027, an mTOR inhibitor (inhibits both mTORC1 and mTORC2 complexes) has been shown to induce protective autophagy in K562 cells and combination of
OSI-027 and CQ-mediated autophagy inhibition resulted in increased apoptosis compared to OSI-027 alone\textsuperscript{108}. Therefore, autophagy may be a key defensive mechanism that provides survival and/or limits the apoptotic responses following mTOR inhibition in CML cells and combined use of potent mTOR inhibitors with autophagy inhibitors may provide an approach to enhance the effect of single drug treatment. In reality, autophagy, a tumor suppressor in normal HSC can be exploited therapeutically in CML.

**Wnt/β-catenin pathway.** Canonical Wnt/β-catenin signaling has been implicated in numerous biological processes in embryogenesis, and hematopoiesis. β-catenin is the critical downstream effector molecule that has an observed role in the proliferation and survival of normal hematopoietic stem cells. Specifically, mice deficient in β-catenin possessed the capacity to form hematopoietic stem cells, but failed in the maintenance of these cells long term\textsuperscript{109}. In CML, the aberrant activation of the Wnt pathway is thought to have a critical role in both disease progression and CML progenitor cell self-renewal and survival. Elevated levels of nuclear β-catenin were detected in the granulocyte-macrophage progenitor pool from patients with blast-phase CML and imatinib-resistant CML compared with marrow collected from healthy individuals\textsuperscript{110}. Implicated in the evolution of CML and progression to the blast phase, BCR-ABL has been observed to directly interact with β-catenin to control levels of protein stabilization and nuclear signaling in malignant cells\textsuperscript{111}. Cross-talk between β- catenin and interferon-regulatory factor 8 (Irf8) as also been shown to affect this progression to blast phase CML, with an Irf8 deletion and constitutive β-catenin activation resulting in the elevated leukemic potential of BCR-ABL+ CML progenitor cells, as well as TKI resistance in the CML murine model\textsuperscript{112}. Deletion of β- catenin in established CML however, was shown to synergize with imatinib to eliminate CML progenitor cells and to delay disease recurrence following discontinuation of TKI treatment *in vivo*\textsuperscript{113}. Pharmacological inhibition of β-catenin via a number of mechanisms in combination with imatinib has produced promising results for the effective
treatment of CML, and eradication of CML progenitor cells.

**TGF-β/FOXO/BCL-6 signalling.** As previously described, the PI3K/Akt pathway is activated in CML cells via BCR-ABL-dependent mechanisms leading to enhanced cellular survival and malignant transformation. In addition, activation of this pathway leads to the phosphorylation, cytoplasmic retention, and inactivation of forkhead O (FOXO) transcription factors. In normal hematopoietic stem cells, FOXOs localize in the nucleus for the regulation of cell proliferation with its transcriptional activity resulting in cell cycle arrest[114]. Loss of functional FOXO has been observed to result in an aberrant increase in ROS production, an increase in the number of stem cells in active cell cycling and eventual population exhaustion. In the BCR-ABL transduction/transplantation CML murine model, active FOXO3a in CML progenitor cells was shown to possess an important role in the maintenance of their self-renewal capacity, with deletion of the transcription factor resulting in increased proliferation, and decreased apoptosis[115](243). In CML CD34+ CML progenitor cells, BCR-ABL expression leads to an increase of FOXO3a in the cytoplasm rendering the factor transcriptionally inactive. Conversely, induction of FOXO3a in leukemic cell lines inhibits cell cycle progression, and induces apoptosis via the activation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and p53[116]. Full restoration of FOXO activity in CML progenitor cells also led to the significant decrease in cyclin D1 mRNA level, and modulation of key target genes including ATM, p57/CDKN1C and BCL-6 which are all required for maintenance of their stem cell-like properties[117,118]. Studies also suggest that FOXOs possess a role in the mechanism of action associated with TKI therapy in CML, with imatinib exposure in several BCR-ABL-expressing cell lines resulting in FOXO3a activation and cell cycle arrest[119]. Interestingly however, is the finding that activation of FOXOs by TKIs is a paradoxical, with signaling also contributing to the protection of CML CML progenitor cells. In general, the anti-proliferative activity of TKIs against CML CD34+ CML progenitor cells is thought to be mediated by the reactivation of FOXO1, 3a, and 4 resulting in quiescence[120].
As an important downstream effector of FOXO, B-cell lymphoma 6 (BCL-6) protein has been identified as a critical factor in the survival and self-renewal of CML progenitor cells through its ability to mediate the repression of Arf and p53. Transforming growth factor-β (TGF-β) has also been implicated in the acquisition of drug resistance in CML progenitor cells via its ability to inhibit Akt activation leading to the release and activation of FOXOs to promote their quiescent properties.

**Sonic hedgehog signaling pathway.** As a critical pathway active during embryogenesis and adult homeostasis, the sonic hedgehog (Shh) pathway is important in the modulation of hematopoietic stem cell proliferation and survival in both physiological and pathological conditions. Briefly, Shh protein ligands bind to Patched (Ptch) leading to the release of the transmembrane protein smoothened (Smo), and a subsequent signal transduction cascade resulting in the nuclear translocation of Gli transcription factors. Multiple studies have indicated that the aberrant activation of the Shh pathway in CML possesses an important role in the regulation of CML progenitor cell survival, as well as in disease progression. The significant upregulation of Shh, Smo, and Gli has been observed in patients with CML compared with normal controls. Loss of functional Smo in the CML murine model led to an impairment of stem cell renewal, a subsequent depletion of these progenitor cells and a subsequent decrease in the induction of disease by BCR-ABL. Conversely, constitutively active Smo increased the number of circulating CML progenitor cells thereby accelerating disease. In addition, pharmacological inhibition of Shh signalling impaired the propagation of BCR-ABL+ CML resistant to imatinib in both murine and human studies. Furthermore, the upregulation of Shh, Smo and Gli-1 protein expression in blast phase CML in comparison to chronic phase disease suggests that the Shh signaling pathway is critical in the transformation and progression of human CML. Most recently, upregulation of Smo was associated with reduced expression
of microRNA-326 in leukemic CD34+ progenitor cells collected from human patients with CML at diagnosis compared to healthy controls\textsuperscript{125}. Taken together, targeting the Shh pathway and its downstream effector proteins may lead to the elimination of disease progenitor cells, with inhibition of Smo or Gli-1 of greatest potential in current research settings.

**Promyelocytic leukemia protein (PML).** The promyelocytic leukemia protein (PML), which is critical in normal hemopoiesis, has been shown to regulate important processes including apoptosis, proliferation, and senescence in hematopoietic stem cells. Deregulation of PML has been observed in CML, with increased expression in bone marrow collected from patients with chronic-phase disease\textsuperscript{118}. Interestingly, an inverse correlation between PML expression and the rates of complete cytogenetic and/or molecular response was determined in these patients, suggesting an important association with prognosis. Ito and co-workers also demonstrated the importance of PML for CML progenitor cell maintenance. Furthermore, treatment of mice with arsenic trioxide, which induces the degradation of PML protein, in combination with chemotherapeutic agent cytarabine, led to the down-regulation of PML and an increase in survival. The administration of arsenic sulfide in combination with imatinib also displayed therapeutic benefit in a BCR-ABL+ murine model\textsuperscript{126}. It was found that while the arsenic sulfide targeted BCR-ABL through ubiquination of important lysine residues leading to proteasomal degradation, imatinib inhibited the PI3K/Akt pathway that ultimately led to cell cycle arrest, decreased tyrosine kinase activity and activation of apoptosis in CML progenitor cells.

**Interferon signaling (IFN).** The role of both IFN-α and IFN-γ in hematopoiesis has led to the investigation of their importance in CML. IFN-γ has been shown to directly induce the rapid expansion of lineage-Sca-1+c-kit+ (LSK) cells, with IFN-γ- induced genes important for hematopoietic stem cell survival and maintenance\textsuperscript{127,128}. Interestingly, evidence suggests a comparable effect of IFN-γ in CML
progenitor cells, with the mediator inducing proliferation in leukemia progenitor cells as well as in primary CD34+ cells collected from newly diagnosed CML patients. In an in vivo model of CML, accelerated disease progression was observed following the secondary transplantation of IFN-γ-treated CML progenitor cells suggesting its importance in the expansion of the population. IFN-α has been shown to activate quiescent hematopoietic stem cells, mediating proliferation. Prior to the discovery of TKI therapies, IFN-α was the standard first-line treatment for CML despite the association with severe adverse effects. Its mechanism of action involves the capacity to restore adhesion of CML progenitor cells to the bone marrow stroma, downregulate the expression of the bcr-abl1 gene, and activate several transcription factors that regulate cell proliferation, maturation, and apoptosis. IFN-α may also induce recognition and elimination of CML cells by the immune system. Most importantly, the chronic administration of IFN-α may lead to a depletion in CML progenitor cells, via direct and indirect mechanisms involving stromal cells. Subsequently, the combination of TKI and IFN-α as a potential treatment approach is hypothesized to synergistically eradicate disease through an ability to target the CML progenitor cell population in disease. Numerous clinical studies have been completed or are in progress to assess the significance of the combinatorial therapeutic strategy, with varying results. All the published trials reported significant incidence of toxicity, with a discontinuation of high dose pegylated IFN-α common during the first year of treatment.

Stromal-derived factor-1 (CXCL12)/ C-X-C chemokine receptor type 4 (CXCR4) axis. The interaction between the chemokine receptor CXCR4 and its ligand CXCL12 is known to be important in early-stage hemopoiesis, and has a critical role in normal hematopoietic stem cell migration. BCR-ABL activity is associated with the down-regulation of CXCR4 expression resulting in the defective adhesion of CML cells to bone marrow stroma mediating the malignant phenotype, and suppressing the CXCL12-induced chemotactic response of CML cells. The CXCR4-dependent migration of immature CD34+ CML cells has
also been shown to be impaired, with the integrin-dependent migration and adhesion in response to CXCL12 significantly decreased\textsuperscript{142}. It is suggested that CML progenitor cells are protected by the bone marrow stroma from imatinib-induced apoptosis, and significantly upregulate the expression of the antiapoptotic factor BCL6 in response to TKI therapy\textsuperscript{143}.

**Protein phosphatase 2A (PP2A).** The inactivation of the tumour suppressor protein PP2A has been observed in CML, via the increased expression of SET, a nucleus/cytoplasm-localized phosphoprotein which is induced by BCR-ABL in a dose- and kinase-dependent manner, progressively increasing during transition to blast phase disease\textsuperscript{144}. BCR-ABL may also inactive PP2A by the induction of Jak2- and/or src-dependent mechanisms\textsuperscript{145}. The activity of PP2A involves the interaction and de-phosphorylation of several factors implicated in the regulation of cell cycle progression, proliferation, survival, and differentiation. Interestingly, several signaling pathways targeted by PP2A phosphatase are also modulated by BCR-ABL, with the expression and/or activity of substrates including myc, STAT5, Akt, BAD and Rb known to be essential in leukemogenesis, found to be altered in blast phase CML\textsuperscript{146}. The inhibition of SET expression or induced expression of PP2Ac in 32D-BCR-ABL cells led to the inhibition of MAPK, STAT5 and Akt phosphorylation, decreased myc expression, and increased levels of pro-apoptotic BAD\textsuperscript{144}. Thus, the suppression of PP2A observed in CML progenitor cells may have a critical role in the prevention of inactivating mitogenic and survival signals.

**Sirtuin-1 (SIRT-1).** The upregulation of the nicotinamide adenine dinucleotide (NAD\textsuperscript{+})-dependent protein deacetylase SIRT-1 through the BCR-ABL-dependent activation of STAT-5 signalling, has been shown to promote cellular survival, and DNA damage repair under oxidative and genotoxic stress\textsuperscript{147}. Yuan and co-workers detected an upregulation in chronic phase CML CD34+ progenitor cells, with a further increase observed in advanced stages. Conversely, normal adult hematopoietic stem cells were shown to express low levels of SIRT-1.
Furthermore, activation of SIRT-1 was associated with the deacetylation of multiple substrates, including FOXO1, p53, and Ku70. In addition, it is suggested that SIRT-1 possesses an important role in the acquisition of TKI-resistant BCR-ABL mutations due to an ability to alter the function of DNA repair mechanisms in CML cells, stimulating activity of error-prone repair following DNA damage$^{148}$. 
2. Rationale of the study

The following considerations have lead the GIMEMA CML WP to design the CML 0811 trial, a phase III-b study with nilotinib 300 mg BID as first-line treatment of chronic-phase CML.

A) Two independent phase 2 studies reported very high rates of cytogenetic and molecular responses in newly diagnosed CML patients treated with nilotinib 400 mg BID\textsuperscript{23,149}. The GIMEMA CML WP trial 0307 reported a 12-month CCyR, MMR, and MR 4.0 rates of 96\%, 85\%, and 7\%, respectively\textsuperscript{23}. A phase 3 randomized study comparing the efficacy and safety of 300 or 400 mg BID nilotinib with imatinib 400 mg daily in early chronic phase CML patients showed the superiority of nilotinib\textsuperscript{25}. Nilotinib 300 mg BID is equally effective and better tolerated than nilotinib 400 mg BID. Based on these results, nilotinib has been registered (FDA and EMA) for the frontline treatment of Ph+ CML at the dose of 300 mg BID. However, nilotinib-associated atherosclerotic adverse events (AAE) were recently uncovered, particularly peripheral arterial occlusive disease\textsuperscript{28,30,31,150,151}, raising concerns on the long-term safety of nilotinib.

Therefore, since it may be possible that nilotinib will be more and more used for the first-line treatment of CML, it is important and useful to explore and to assess the efficacy and safety of nilotinib in a multicenter, independent study of an unselected cohort of patients.

B) Currently, the most important target of the treatment of CML with TKIs is the deep MR, marker of better long-term outcome. Patients with CML on IFN therapy that achieve a ratio of BCR-ABL/ABL transcripts of 0.05\% or less have an excellent long-term prognosis\textsuperscript{152}. Another study published by the MDACC reported that achieving a MMR on imatinib correlates with an improved probability of a durable cytogenetic remission\textsuperscript{153}. Results from the IRIS study suggest that obtaining the MMR after 12 months of imatinib therapy may be a marker of stable response. Further on, in the same study, patients with a MMR after 12 months of therapy had a significantly better probability of disease-free
survival compared with those in complete cytogenetic remission, but without a MMR\textsuperscript{154}.

C) Deep MR as been identified as a prerequisite to treatment discontinuation: few experiences have been published aimed to evaluate the persistence of the MR after imatinib discontinuation. In a pilot study\textsuperscript{155}, 12 patients discontinued imatinib after at least 2 years of MR 5.0 (median duration of negativity, 32 months). Six patients displayed a molecular relapse with a detectable BCR-ABL transcript at 1, 1, 2, 3, 4, and 5 months. Imatinib was then reintroduced and led to a novel molecular response. Six other patients (50\%) still have an undetectable level of BCR-ABL transcript after a median follow-up of 18 months (range, 9-24 months). The results of this pilot trial have been confirmed and extended in a second trial, the STIM trial, published in 2010\textsuperscript{156}. One hundred patients were enrolled, and 69 patients had at least 12 months follow-up: 42 (61\%) of these 69 patients relapsed (40 before 6 months, one patient at month 7, and one at month 19). At 12 months, the probability of persistent MR 5.0 for these 69 patients was 41\% (95\% CI 29–52). All patients who relapsed responded to reintroduction of imatinib. In another study on 80 patients who stopped TKIs after a prolonged deep MR (MR 4.5 – 5), 29 patients (36\%) lost MMR after a median of 4 months off therapy (range, 2 to 17 months); Authors identified the loss of MMR as a safe condition for restarting treatment with TKIs.

Higher rates of deep MR induced by nilotinib compared to imatinib could translate in a higher proportion of patients candidate to stopping anti-CML treatment, with higher probability of remaining disease-free in the long term. The advantages of this possible future scenario could be: first, the chance of treatment discontinuation, at least in patients with chronic clinical adverse events; second, a potential reduction of the costs of TKI treatment (after the introduction of TKI, with the increasing prevalence of CML patients, the costs of CML treatment is increasing year by year).

D) The relapses observed in patients with a stable deep molecular response, together with several biologic studies showing the persistence of leukemic stem cells in patients with deep molecular response, suggest that targeting the
leukemic stem cells would be useful for the eradication of the disease. However, several mechanisms could be implicated in leukemic stem cells persistence, and probably most of them are similar to those that are active in normal stem cells. Therefore, the characterization of the leukemic stem cells and the understanding of their differences with the normal counterpart are of utmost importance.

Based on these considerations, the aim of the current study is to assess the efficacy, mainly in terms of deep MR rates, of nilotinib 300 mg BID treatment in newly diagnosed CML-CP patients, in an independent, investigator sponsored study. Monitoring of molecular response provides a straightforward opportunity to assess patients' response and possible prognosis in the use of targeted therapy. The most important endpoint of the present study is the rate of MR 4.0 at 24 months, which will provide important information to plan and perform future studies aimed to discontinue the treatment, either primarily or after addition of other drugs targeting residual leukemic stem cells. Translational studies are conducted on leukemic stem cells, in order to identify those characteristics that may promote the persistence of the disease despite TKI treatment.
3. Methods

3.1. Objective of the trial

3.1.1. Primary objective
The primary objective of the CML 0811 trial is to evaluate the efficacy of nilotinib, 300 mg twice daily with dose increase to 400 mg twice daily in case of suboptimal response or failure\(^\text{11}\) (excluding patients who will fail for progression to AP/BP), in a population of patients with Ph-positive, BCR-ABL positive CML in early CP.

3.1.2. Secondary Objectives
- the assessment of the treatment safety;
- the evaluation of complete cytogenetic response (CCyR) rates and kinetics of molecular response;
- the estimation of the overall survival (OS), the progression-free survival (PFS), the failure-free survival (FFS) and the event-free survival (EFS);
- the characterization of the leukemic stem cells at the proteomic level

3.2. Trial Design

3.2.1 Patient selection criteria

Inclusion Criteria
- Age $\geq$ 18
- Male or female patients with diagnosis of Ph+ and/or BCR-ABL+ CML
- Early chronic phase (within 6 months from diagnosis)
- Pretreatment with Hydroxyurea or Anagrelide for a duration of up to 3 months and/or pretreatment with Imatinib for up to 30 days are permitted
- Normal serum levels of potassium, magnesium, phosphorus, total calcium corrected for serum albumin or phosphorus, or correctable to within normal limits with supplements prior to the first dose of study medication
• Written informed consent prior to any study procedures being performed
• AST and ALT ≤ 2.5 x ULN or ≤ 5.0 x ULN if considered due to leukemia
• Alkaline phosphatase ≤ 2.5 x ULN unless considered due to leukemia
• Total direct bilirubin ≤ 1.5 x ULN, except known Mb. Gilbert
• Serum creatinine ≤ 1.5 x ULN

Exclusion criteria
• Known impaired cardiac function, including any of the following:
  • LVEF < 45%
  • Complete left bundle branch block
  • Right bundle branch block plus left anterior hemiblock, bifascicular block
  • Use of a ventricular-paced pacemaker
  • Congenital long QT syndrome
  • History of or presence of clinically significant ventricular or atrial tachyarrhythmias
  • Clinically significant resting bradycardia (<50 beats per minute)
  • QTc>450 msec on screening ECG. If QTc > 450 msec and electrolytes are not within normal ranges before Nilotinib dosing, electrolytes should be corrected and then the patient rescreened for QTc criterion.
• Myocardial infarction within 12 months prior to starting study drugs
• Other clinical significant heart disease (e.g. unstable angina, congestive heart failure, uncontrolled hypertension)
• Serum lipase and amylase > 1.5 x ULN (upper limit of normal) or history of acute (i.e., within 1 year of starting study medication) or chronic pancreatitis
• Other concurrent uncontrolled medical conditions (e.g., uncontrolled diabetes, active or uncontrolled infections, acute or chronic liver and renal disease) that could cause unacceptable safety risks or compromise compliance with the protocol
• Impaired gastrointestinal function or disease that may alter the absorption of study drug (e.g., ulcerative disease, uncontrolled nausea, vomiting and
diarrhea, malabsorption syndrome, small bowel resection or gastric bypass surgery)

- Concomitant medications with potential QT prolongation (see link for complete list: http://www.torsades.org/medical-pros/drug-lists/printable-drug-list.cfm)

- Concomitant medications known to interact with CYP450 isoenzymes (CYP3A4, CYP2C9, and CYP2C8: see link for complete list (http://medicine.iupui.edu/flockhart/table.htm)

- Patients who have undergone major surgery ≤ 2 weeks prior to starting study drug or who have not recovered from side effects of such therapy

- Patients who are pregnant or breast feeding, or women of reproductive potential not employing an effective method of birth control. (Women of childbearing potential must have a negative serum pregnancy test within 48 hours prior to administration of nilotinib).

- Patients with a history of another primary malignancy that is currently clinically significant or currently requires active intervention.

- Patients unwilling or unable to comply with the protocol.

3.2.2. Treatment scheme

This study is an open-label, multicenter, phase III-b study of nilotinib administered orally at the dose of 300 mg twice daily (total daily dose 600 mg daily) for 24 months (study core), and indefinitely if it is in the interest of the patient. Nilotinib dose is increased to 400 mg BID in case of suboptimal response or failure, according to ELN 2009 recommendations, with the exception of patients who will fail for progression to AP/BP: in this case the patient will not be treated with study drug and the choice of the treatment will be up to the physician (Figure 1).

Study duration is estimated in 6 years, 1 year of estimated enrollment, 2 years therapy duration. Thereafter, information is due for other 3 years.
The main data analysis will be performed when all patients will complete 24 months of treatment (or discontinued earlier). Safety and tolerability profile will be assessed by collecting hematologic and non-hematologic adverse events, laboratory examinations and ECG data. The molecular response will be assessed using the GIMEMA standardized molecular laboratories (Labnet network).

**Figure 1. Study flow-chart**

*Complete Response HR (CHR) definition*  
- WBC < 10x10⁹/L  
- Platelet count < 450x10⁹/L  
- No immature granulocytes in differential  
- Basophils less than 5%  
- Spleen non palpable  

*Hematologic response loss definition*  
- the loss of any of the criteria which define CHR

**Definition of failure,**  
- No CHR at 3 months  
- No CgR (Ph pos 95%) at 6 months  
- Less than PCgR at 12 months  
- Less than CCgR at 18 months  
- Loss of CHR, any time  
- Loss of CCgR, any time  
- Clonal Chromosomal Abnormalities (CCA) in Ph+ cells.  
- BCR-ABL kinase domain mutations, any time
3.2.3. Primary endpoint
To assess the deep molecular response (MR 4.0) rate at 24 months of treatment. For the purpose of this protocol, MR 4.0 is defined as a BCR-ABL/ABL transcripts ratio ≤ 0.01%, or undetectable BCR-ABL transcripts, with quantitative RT-PCR in a peripheral blood sample of at least 10 ml, with a at least 10,000 copies of the control gene ABL<sup>14</sup>.

3.2.4. Secondary endpoints
- The toxicity and the compliance to treatment
- The complete cytogenetic response (CCgR) rate at 3, 6, 12, 18, 24 and 60 months;
- The rate and the degree of molecular response at 3, 6, 12, 18, 24 and 60 months;
- The time to CCgR, the time to MMR and the time to MR 4.0;
- Overall Survival (OS), from the date of the first nilotinib dose to death, Progression Free Survival (PFS), from the date of the first nilotinib dose to progression to AP or BP or death, Failure Free Survival (FFS) from the date of the first nilotinib dose to failure or progression or death, Event Free Survival (EFS) from the date of the first nilotinib to any event, including treatment discontinuation for adverse events, failure, progression to AP or BP, or death, whichever comes first. Failure is defined according to ELN 2013 criteria<sup>18</sup>.

3.2.5. Therapeutic regimens, expected toxicity, dose modifications

Drug information
Investigational treatment: nilotinib (AMN107, Tasigna, Novartis Pharma).

Dose and schedule
Patients will be treated with nilotinib:
- From day 1 onward, 300 mg orally each morning and evening approximately 12 hours apart (300 mg BID).
- In case of suboptimal response or failure (excluding progression to AP/BP) and in absence of safety concerns at 300mg BID, the dose is increased to 400 BID for 3 months; after 3 months, Nilotinib is continued at 400 BID in case of optimal or suboptimal response and Nilotinib is discontinued in case of failure.
- After 3 additional months at 400 mg BID, patients still suboptimal may continue at the same dose but after agreements with the Treatment Monitoring Committee.

Therapy with nilotinib will be continued for up to 24 months, until progression to AP/BP or the development of intolerance of treatment. After 24 months of treatment, all the patients who at 24 months are in MR 4.0, will be offered to continue the study drug free-of-charge until the responsible physician and the patient will consider this in the best interest of the patient. For all the patients who will not be in MR 4.0 at 24 months, the choice of the treatment will be up to the physician. However, all the patients, whether or not in MR 4.0, will be followed-up for at least 5 years, and will be offered to participate in subsequent studies of the GIMEMA CML WP.

Dose and schedule modifications
Subjects will be monitored continuously for adverse events (toxicity) while on study therapy. This study uses the CTCAE (NCI Common Terminology Criteria for Adverse Events) version 4.0 for toxicity and AE reporting. The highest reported AE grade should be used to determine the dose modification action.

Dose reduction guidelines for study drugs-related non-haematologic toxicity
Grade ≥ 2 non-haematologic toxicity must be resolved within 28 days in order to resume study drug at the reduced dose. If a grade ≥ 2 non-haematologic toxicity does not resolve after 28 days of study drug interruption, the continuation of the
treatment must be discussed by Local Investigator and Treatment Monitoring Committee (TMC) to decide if continuing therapy is in the patient's best interest.

This applies to all clinical AEs, with the exception of cardiac AEs which have specific guidelines and to biochemical AEs with the exception of transaminases, bilirubin, lipase and amylase for which specific recommendations are made, aimed to keep the treatment at the same dose in case of grade 2 AEs.

3.2.6. Clinical evaluation, laboratory tests and follow-up
A patient can be enrolled within 6 months from diagnosis. Pretreatment with Hydroxyurea or Anagrelide for a duration of up to 3 months and/or pretreatment with Imatinib for up to 30 days are permitted. Patients can be enrolled only if spleen size, peripheral blood percentage of blast cells, eosinophils and basophils, and platelet count have been recorded prior to any antileukemic treatment, because these data are necessary for assessing the risk.

Before treatment starts
- Inclusion/exclusion criteria
- Informed consent
- Symptoms
- Physical examination (height, weight, spleen, in cm below costal margin, vital signs and other relevant findings, including pulse rate and blood pressure)
- Bone Marrow (BM) aspirate for cytogenetics, qualitative and mutational analysis (chromosome banding analysis is mandatory;). A sample must be stored for mutational analysis.
- Blood counts and differential
- Serum protein concentration and electrophoretic profile, IgG, IgA, IgM concentration
• Serum chemistry (BUN, creatinine, AST, ALT, ALP, GT, bilirubine, glycemia, glycated hemoglobin, amylase, lipase, Na, K, Ca, P, Cl and Mg, cholesterol, HDL cholesterol, LDL cholesterol, triglycerides)
• Pregnancy test
• Peripheral Blood (PB) for the assessment of BCR-ABL level (RT-Q-PCR). Optional if performed at diagnosis.
• A PB sample for quantitative molecular analysis (RT-Q-PCR) and FISH
• Peripheral blood for translational studies

Before treatment starts - BASELINE (day 1)
• Demography
• Medical history
• Current medical conditions
• Prior and concomitant medications
• Symptoms
• Physical examination (height, weight, spleen, in cm below costal margin, vital signs and other relevant findings, including pulse rate and blood pressure)
• 12-leads ECG and Echocardiogram

During treatment (first 24 months)
• Physical examination (spleen, in cm below costal margin, vital signs and other relevant findings, including body weight, pulse rate and blood pressure), is due every 15 day for three months, hence every 3 months.
• Symptoms and adverse events are recorded continuously
• Concomitant medications and nilotinib dosing are recorded continuously
• Blood counts and differential are due at least every 15 days for the first 3 months, hence at least every month until month 6 and at least every 3 months thereafter.
• Serum protein concentration and electrophoretic profile, IgG, IgA, IgM concentration are due every 6 months.
• Serum chemistry is due to every 15 days for 3 months, hence every month until month 6 and every 3 months thereafter.
• A 12 lead ECG is due after 1, 3, 6 and 12 and 24 months.
• A BM aspirate for cytogenetics (chromosome banding analysis) is due after 3, 6, and 12, 18 and 24 months.
• A PB sample for quantitative molecular analysis (RT-Q-PCR) is collected at 3, 6, 9, 12, 15, 18 and 24 months. FISH is optional baseline, due at 3, 6, 12, 18 and 24 months in case of insufficient n. of metaphases (< 20) for BM cytogenetic evaluation

**During treatment (from 24 to 60 months)**
The assessment is due for three years and includes:
• Physical examination every 6 months
• Symptoms every 6 months
• Concomitant medications, nilotinib dosing, and adverse events are recorded continuously
• Blood counts and differential every 6 months
• Serum protein concentration and electrophoretic profile, IgG, IgA, IgM concentration every 6 months
• Serum chemistry every 6 months
• PB sample for quantitative molecular analysis (RT-Q-PCR) will be collected every 3 months. A BM aspirate for cytogenetics (chromosome banding analysis) as per clinical practice
• 12 lead ECG and echocardiogram are due every 12 months
• Any SAE must be communicated immediately also after the end of study core.

**End of treatment**
At any time in case of treatment failure or treatment discontinuation:
• Physical examination (spleen in cm below costal margin, vital signs and other relevant findings, including body weight, pulse rate and blood pressure)
• Symptoms and adverse events
• Concomitant medications and nilotinib dosing.
• Blood counts and differential
• Serum protein concentration and electrophoretic profile, IgG, IgA, IgM concentration
• Serum chemistry
• 12 lead ECG
• BM aspirate for cytogenetics (chromosome banding analysis) and mutational analysis. In case of blast crisis, PB can substitute for BM.
• PB sample for quantitative molecular analysis (RT-Q-PCR)

3.2.7. Criteria of evaluation

Definition of chronic phase (CP)
All the following criteria:
- less than 15% blasts in blood (marrow)
- less than 30% blasts + promyelocytes in blood (marrow)
- less than 20% basophils in blood (marrow)
- no extrahematologic involvement, apart from spleen and liver

Definition of accelerated phase (AP)
Any of the following criteria:
- more than 15% but less than 30% blasts in blood (marrow)
- more than 30 % but less than 50% blasts + promyelocytes in blood (marrow)
- more than 20% basophils in blood (marrow)

Definition of blast phase (BP)
Any of the following criteria:
- more than 30% blasts in blood (marrow)
- more than 50% blasts + promyelocytes in blood (marrow)
- any extrahematologic involvement, apart from spleen

**Definition of risk**
For risk definition, the following criteria are required at diagnosis, prior to any treatment:
- Age
- Spleen size (cm below costal margin – max distance)
- Platelet count
- Blood blasts %
- Blood eosinophils %
- Blood basophils %

The risk is calculated according to the international prognostic formulations, Sokal\(^{157}\), EURO\(^{158}\), and EUTOS\(^{159}\).

**Definition of failure**
The definition of failure, adapted to the European LeukemiaNet 2013 recommendations\(^{18}\), includes:
- No CHR and/or no CgR (Ph+ > 95%) at 3 months
- Less than PCgR at 6 months and/or BCR-ABL >10%
- Less than CCgR at 12 months and/or BCR-ABL >1%
- Loss of CHR, any time
- Loss of CCgR, any time
- Confirmed loss of MMR, any time
- Clonal Chromosomal Abnormalities (CCA) in Ph+ cells, any time.
- BCR-ABL kinase domain mutations, any time

**Complete HR (CHR)**
Complete HR (CHR) is defined by all the following criteria:
- WBC < 10x10^9/L
- Platelet count < 450x10^9/L
- No immature granulocytes in differential
- Basophils less than 5%
- Spleen non palpable
- Hematologic response loss is defined by the loss of any of the criteria which define CHR

**Cytogenetic response**
Conventional Banding Analysis (CBA) is performed on bone marrow cells after short term culture (24 and/or 48 hours). The cells are treated with colchicine and with hypotonic solution. The pellet is fixed and washed in methanol-acetic acid (3:1). The cells are resuspended in fixative and dropped on slides. Karyotypes are examined after G-banding or Q-banding.
The minimum number of metaphases to be scored is 20.
CBA is performed with the purpose to evaluate:
- the proportion of Ph pos metaphases
- the presence of variant translocations
- the presence of clonal chromosome abnormalities (CCA) in Ph pos cells
- the presence of clonal chromosome abnormalities (CCA) in Ph neg cells
All the cytogenetic abnormalities are captured and reported in the e-CRF.

The CgR is defined based on the percentage of Ph pos metaphases, as evaluated by chromosome banding analysis of at least 20 marrow cell metaphases:
- Complete (CCgR) if Ph pos 0
- Partial (PCgR) if Ph pos 1-34%
- Minor (mCgR) if Ph pos 35-65%
- Minimal or none (min/none CgR) if Ph pos > 65%
If only interphase FISH data from PB are available, the response can be defined only as non-complete or complete – to be complete by FISH, it is required that less than 1% of cells (minimum number 200) have a positive signal.

CgR loss is defined whenever a CCgR is lost to PCgR or less.
Clonal Chromosome Abnormalities (CCA)
CCA are defined by any chromosome abnormality that becomes detectable in at least two cells in two subsequent cytogenetic examinations.

Fluorescence-in-situ-hybridization (FISH)
FISH analysis of PB cells is required if marrow cells cannot be obtained, at any time point. FISH is performed on the cytogenetic pellet of blood cells or marrow, using DNA probes that hybridizes to BCR and ABL regions. Only Extra-Signal (ES), Dual-Color Dual-Fusion (DCDF) or D-FISH BCR-ABL commercial probes can be used.

Molecular Response (MR)
RT-Q-PCR of BCR-ABL transcripts will be performed with the TaqMan technology as previously set up and standardized within the framework of the Europe Against Cancer (EAC) program ABL will be used as control gene to compensate for differences in RNA quality or RT efficacy, and results will be expressed as ratio of BCR-ABL/ABL%.

The molecular response is assessed on peripheral blood samples using RT-Q-PCR and is expressed as a ratio between BCR-ABL and ABL:

- Major Molecular Response (MMR) is defined as a BCR-ABL/ABL ratio lower than 0.10% (as corrected by lab conversion factor, according to the international scale).
- Deep Molecular Response: 2 different degree and deepness of response will be analyzed, the MR 4.0, which defines the primary endpoint and the MR 4.5
- MR 4.0 is defined as an undetectable BCR-ABL transcripts level, or detectable BCR-ABL transcripts with BCR-ABL/ABL ratio < 0.01%, with a sensitivity of at least 10.000 copies of ABL.
- MR 4.5 is defined as an undetectable BCR-ABL transcripts level, or detectable BCR-ABL transcripts with BCR-ABL/ABL ratio < 0.0032%, with a sensitivity of at least 32,000 copies of ABL.

3.2.8. Statistical methods and data analysis

Sample size
Currently, the most important target of the CML therapy is the deep molecular response, a pre-requisite for treatment discontinuation. The aim of the current study is to investigate the deep MR rates of nilotinib. The primary endpoint is the MR 4.0 rate at 24 months. Sample size estimation has been performed using a two-sided binominal test. The MR 4.0 rate at 24 months during imatinib therapy is approximately 10%. Establishing that the minimum rate of interest with nilotinib is 20%, a sample size of 109 patients is required for a 85% power (1-beta) and a two-sided type 1 error alfa of 5%. Assuming a 1% of patients with atypical BCR-ABL transcript (not evaluable for molecular response) and a 10% drop-out rate, the total number of patients becomes 122. Patients dropping out early or not providing sufficient or missing data for any other reason will be included in the analysis set as non-responders. A minimum of 19 patients with MR 4.0 at 24 months should be observed to define a study success.

Populations for analysis
This study follows an open-label, single-arm, multi-center design. It is planned that the data from all centers that participate in this protocol will be pooled and utilized. The main analysis will be performed when the last patient completes the 24-month visit. ITT analysis set: all patients who received at least one dose of study drug. The ITT analysis set will be used for all safety and efficacy analyses.

Statistical analysis
Patient demographics/baseline characteristics: Qualitative data will be summarized by means of contingency tables and quantitative data will be summarized by appropriate descriptive statistics.
Cytogenetic response will be calculated for the Ph positive patients only. Patients with atypical transcripts will not be considered for the molecular analysis. The cytogenetic and molecular response rates at each time-point will be calculated according to the ITT principle (ITT analysis set). Time-to-response is defined as: [date of first response - date of first study drug administration +1]. All the time-to-response variables will be calculated using Kaplan Meier’s product limit estimates. The corresponding 95% confidence intervals will be computed as well. OS, PFS, FFS and EFS will be calculated from the date of start of treatment until death (OS), progression or death (PFS), failure or death (FFS), or any event (EFS), whichever comes first. Failure is defined according to ELN 2013 criteria\textsuperscript{18}. For overall survival and progression-free survival, follow-up data after the discontinuation of the treatment will also be included. All the time to event variables will be calculated using Kaplan Meier’s product limit estimates. The corresponding 95% confidence intervals will be computed as well.

The assessment of safety will be based mainly on the frequency of AEs, laboratory abnormalities and clinically notable ECG data. All AEs and laboratory abnormalities recorded during the study will be summarized by system organ class, severity (based on CTC v 4.0 AE grades) and type of AE. The duration of exposure to the study medication and total doses taken by the patient will be summarized.

Deaths reportable as SAEs and non-fatal SAEs will be listed by patient and tabulated by type of AE. Other safety data (e.g., vital signs, special tests) will be considered as appropriate.

3.3. Leukemic Stem cell collection and proteomic analysis

CD34+ cells were purified by immune-magnetic separation from peripheral blood (PB) of 7 newly diagnosed chronic phase (CP) CML patients and compared to the normal counterpart obtained from normal bone marrow of three healthy donors (NBM) and/or from umbilical cord blood (CB) of three donors.
The phosphorylation status of 40 different proteins belonging to numerous signal-transduction pathways, and the expression of 31 proteins of the apoptotic machinery, were assessed using a customized direct phase proteome profiler antibody array. The resulting dots were visualized using ECL and quantified by densitometric analysis.

**Figure 2. List of proteins and corresponding phosphorylation sites detected in the study**

<table>
<thead>
<tr>
<th>Phospho-proteins</th>
<th>Apoptosis and cell cycle related proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPKs family</td>
<td>Bcl-2 family</td>
</tr>
<tr>
<td>p38α (T180/Y182)</td>
<td>Bad</td>
</tr>
<tr>
<td>ERK1/2 (T202/Y204, T185/Y187)</td>
<td>Bax</td>
</tr>
<tr>
<td>RSK1/2/3 (S380/S386/S377)</td>
<td>Bcl-2</td>
</tr>
<tr>
<td>CREB (S133)</td>
<td>Bcl-x</td>
</tr>
<tr>
<td>JNK pan (T183/Y185, T221/Y223)</td>
<td>TRAIL R1/DR4</td>
</tr>
<tr>
<td>c-Jun (S63)</td>
<td>TRAIL R2/DR5</td>
</tr>
<tr>
<td>MSK1/2 (S376/S360)</td>
<td>FADD</td>
</tr>
<tr>
<td>PI3K pathway</td>
<td></td>
</tr>
<tr>
<td>AKT (S473)</td>
<td></td>
</tr>
<tr>
<td>AKT (T308)</td>
<td>Fas/TNFRSF6</td>
</tr>
<tr>
<td>mTOR (S2448)</td>
<td></td>
</tr>
<tr>
<td>p70 S6 Kinase (T389)</td>
<td></td>
</tr>
<tr>
<td>p70 S6 Kinase (T421/S424)</td>
<td></td>
</tr>
<tr>
<td>PLCγ-1 (Y783)</td>
<td></td>
</tr>
<tr>
<td>STATs family</td>
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<tr>
<td>STAT2 (Y689)</td>
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<tr>
<td>STAT3 (Y705)</td>
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</tr>
<tr>
<td>STAT5a (Y694)</td>
<td></td>
</tr>
<tr>
<td>STAT5b (Y699)</td>
<td></td>
</tr>
<tr>
<td>STAT5a7b (Y694/Y699)</td>
<td></td>
</tr>
<tr>
<td>STAT6 (Y641)</td>
<td></td>
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<tr>
<td>p53</td>
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<td>p53 (S392)</td>
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<td>p53 (S46)</td>
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<tr>
<td>p53 (S15)</td>
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</tr>
<tr>
<td>Tyrosine kinases</td>
<td></td>
</tr>
<tr>
<td>GSK-3α/β (S21/579)</td>
<td></td>
</tr>
<tr>
<td>Src (Y418)</td>
<td></td>
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<tr>
<td>Lyn (Y397)</td>
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<td>Lck (Y394)</td>
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<td>Fyn (Y420)</td>
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<td>Yes (Y426)</td>
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<tr>
<td>Fgr (Y412)</td>
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<tr>
<td>Hck (Y411)</td>
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<tr>
<td>Chk-2 (T68)</td>
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</tr>
<tr>
<td>FAK (Y397)</td>
<td></td>
</tr>
<tr>
<td>Cell cycle inhibitors</td>
<td></td>
</tr>
<tr>
<td>Cell growth, adhesion and migration</td>
<td></td>
</tr>
<tr>
<td>p27 (T198)</td>
<td></td>
</tr>
<tr>
<td>β-Catenin</td>
<td></td>
</tr>
<tr>
<td>Cellular checkpoint</td>
<td></td>
</tr>
<tr>
<td>phospho-Rad17 (S635)</td>
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</tr>
<tr>
<td>Molecular chaperones</td>
<td></td>
</tr>
<tr>
<td>HSP27 (S78)</td>
<td></td>
</tr>
<tr>
<td>HSP70 (S82)</td>
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</tr>
<tr>
<td>Homeostasis sensors</td>
<td></td>
</tr>
<tr>
<td>AMPKα1 (T174)</td>
<td></td>
</tr>
<tr>
<td>AMPKα2 (T172)</td>
<td></td>
</tr>
<tr>
<td>AMPKβ2 (S177)</td>
<td></td>
</tr>
<tr>
<td>Ion (Ca++) channel regulator</td>
<td></td>
</tr>
<tr>
<td>Pyk2 (Y402)</td>
<td></td>
</tr>
<tr>
<td>Apoptosis Inhibitors</td>
<td></td>
</tr>
<tr>
<td>XIAP</td>
<td></td>
</tr>
<tr>
<td>Survivin</td>
<td></td>
</tr>
<tr>
<td>Apoptosis activators</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c</td>
<td></td>
</tr>
<tr>
<td>SMAC/Diablo</td>
<td></td>
</tr>
<tr>
<td>Molecular chaperones</td>
<td></td>
</tr>
<tr>
<td>HO-1/HMOX1/HSP32</td>
<td></td>
</tr>
<tr>
<td>HO-2/HMOX2</td>
<td></td>
</tr>
<tr>
<td>HSP27</td>
<td></td>
</tr>
<tr>
<td>HSP60</td>
<td></td>
</tr>
<tr>
<td>HSP70</td>
<td></td>
</tr>
<tr>
<td>HTRA2/Omi</td>
<td></td>
</tr>
<tr>
<td>Clusterin</td>
<td></td>
</tr>
<tr>
<td>ROS scavenging</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
</tr>
<tr>
<td>PON2</td>
<td></td>
</tr>
<tr>
<td>Cell cycle Inhibitors</td>
<td></td>
</tr>
<tr>
<td>p21/CIP1/CDKN1A</td>
<td></td>
</tr>
<tr>
<td>p27/Kip1</td>
<td></td>
</tr>
<tr>
<td>Cellular checkpoint</td>
<td></td>
</tr>
<tr>
<td>Claspin</td>
<td></td>
</tr>
<tr>
<td>Transcription factor</td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td></td>
</tr>
</tbody>
</table>
4. Results

4.1. Clinical results

4.1.1. Patients characteristics

One hundred thirty patients were enrolled in 33 Centers in Italy. The median age was 50 years (range 18-85); 19% of the patients were ≥ 65 years old; 66% of the patients were males. At baseline, 5% of the patients had clonal cytogenetic abnormality in the Philadelphia-positive cells (CCA Ph). Variant translocations were observed in 7% of the patients.

The proportion of low risk patients according to Sokal, EURO, and EUTOS scores was 43%, 50%, and 92% respectively. The proportion of intermediate risk patients according to Sokal and EURO score was 37% and 45%; the proportion of patients with high-risk score was 20%, 5%, and 8%, respectively.

The median follow-up at the cut-off of the present analysis was 24 months (range 18-36). Patients characteristics are summarized in table 4.

Table 4. Patients Characteristics

<table>
<thead>
<tr>
<th>Patients, n</th>
<th>130</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years; median (range)</td>
<td>50 (18-85)</td>
</tr>
<tr>
<td>65 years or older; n (%)</td>
<td>25 (19)</td>
</tr>
<tr>
<td>Sex male, n (%)</td>
<td>86 (66)</td>
</tr>
<tr>
<td>Cytogenetics; n (%)</td>
<td></td>
</tr>
<tr>
<td>CCA Ph+</td>
<td>7 (5)</td>
</tr>
<tr>
<td>Variant translocations</td>
<td>9 (7)</td>
</tr>
</tbody>
</table>
### 4.1.2 Molecular response

The primary end-point of the trial was the rate of MR 4.0 at 24 months. Secondary end-points included the analysis of the rates of MR 4.5. For these analyses, the availability of samples with adequate sensitivity is of utmost importance. In fact, the definition of the different levels of deep molecular response, according to international accepted criteria\textsuperscript{14}, is:

- **MR 4.0:** detectable disease \( \leq 0.01\% \) BCR-ABL or undetectable disease, with \( \geq 10,000 \) ABL copies
- **MR 4.5:** detectable disease \( \leq 0.0032\% \) BCR-ABL or undetectable disease, with \( \geq 32,000 \) ABL copies
- **MR 5.0:** detectable disease \( \leq 0.001\% \) BCR-ABL or undetectable disease, with \( \geq 100,000 \) ABL copies

Considering all the samples with a BCR-ABL/ABL ratio < 0.1\% (corresponding to MMR), 90\% of them had a number of copies of the control gene > 10,000, therefore appropriate to define this level of response. More in detail, 52\% of the samples had a number of ABL copies of 10,001 – 31,999, therefore able to define a MR 4.0; 27\% had a number of copies of ABL of 32,000 – 99,999, therefore enough to define a MR 4.5; 10\% of the samples had a number of copies > 100,000, therefore able to define a MR 5.0 (Table 5).
The molecular response at each time point is illustrated in figure 3. The analysis is according to the intention-to-treat principle, therefore, not evaluable samples are considered as non-responders.

At 3 months the rates of MMR, MR 4.0, and MR 4.5 were 18%, 3%, and 0%, respectively; At 6 months the rates of MMR, MR 4.0, and MR 4.5 were 53%, 12%, and 2%, respectively; At 12 months the rates of MMR, MR 4.0, and MR 4.5 were 57%, 28%, and 7%, respectively; At 18 months the rates of MMR, MR 4.0, and MR 4.5 were 65%, 29%, and 11%, respectively.

The median time to major molecular response was 6 months.

The estimated 24 months rates of MR 4.0 and MR 4.5 were 47% (38 – 57%) and 22% (16 – 31%) (Figure 4).

The stability of the molecular response was also analyzed. Sustained MR 4.0 (4.5) was defined as MR 4.0 (4.5) for at least 1 year, with at least 3 evaluable analyses. Overall, 57/130 (44%) patients achieved a MR 4.0; 27/57 (47%, or 21% of the total) had a sustained MR 4.0; 30/130 (23%) patients achieved a MR 4.5; 6/30 (20%, or 5% of the total) had a sustained MR 4.5.

<table>
<thead>
<tr>
<th>Number of ABL copies</th>
<th>Percentage of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10.000</td>
<td>10%</td>
</tr>
<tr>
<td>10.000 - 31.999 (MR^4.0)</td>
<td>52%</td>
</tr>
<tr>
<td>32.000 - 99.999 (MR^4.5)</td>
<td>27%</td>
</tr>
<tr>
<td>&gt; 100.000 (MR^5.0)</td>
<td>10%</td>
</tr>
</tbody>
</table>
Figure 3. Molecular response at each time-point

Figure 4. Estimated 24 months cumulative rates of MR 4.0 and MR 4.5
4.1.3. Response according to ELN 2013 recommendations

The response at the 3rd month, according to the cytogenetic evaluation, was classified as optimal, warning, failure, or not evaluable in 61.5%, 3%, 0.7%, and 34.6% of the patients, respectively. According to the molecular evaluation it was classified as optimal, warning, or not evaluable in 76.9%, 4.6%, and 18.4% of the patients, respectively.

The response at the 6th month, according to the cytogenetic evaluation, was classified as optimal, warning, failure, or not evaluable in 70.8%, 1.5%, 3%, and 24.6% of the patients, respectively. According to the molecular evaluation it was classified as optimal, warning, failure, or not evaluable in 73.1%, 6.9%, 2.3, and 18.4% of the patients, respectively.

The response at the 12th month, according to the cytogenetic evaluation, was classified as failure, or not evaluable in 1.5%, and 20.7% of the patients, respectively. According to the molecular evaluation it was classified as optimal, warning, failure, or not evaluable in 53.1%, 19.2%, 1.5%, and 25.4% of the patients, respectively.

Figure 5. Response at different time-points according to ELN 2013 recommendations
Combining the hematologic, cytogenetic, and molecular data (Figure 5), the response at the 3rd month was classified as optimal, warning, failure, or not evaluable in 82.3%, 6.1%, 0.7%, and 10.7% of the patients, respectively. At the 6th month, it was classified as optimal, warning, failure, or not evaluable in 80%, 6.9%, 3.8%, and 9.2% of the patients, respectively. At the 12th month, it was classified as optimal, warning, failure, or not evaluable in 53.1%, 18.4%, 2.3%, and 25.4% of the patients, respectively.

4.1.4. Events
Overall, events that lead to permanent nilotinib discontinuation were recorded in 23 patients (18.4%). Failures according to ELN 2013 criteria were observed in 7 (5.3%) patients, and progression to accelerated/blast phase in 2 (1.5%) patients. At last contact, all patients were alive.

The estimated 24-months event-free survival (EFS), failure-free survival (FFS), progression-free survival (PFS), and overall survival (OS), were 74%, 93%, 98%, and 100%, respectively (Figure 6).

Figure 6. Estimated 24-months survival measures
4.1.5. Cardiovascular events

Seven cardiovascular events were observed (Table 6). In 5 cases, they had an atherosclerotic pathogenesis: 3 coronary artery diseases and 2 peripheral arterial thrombosis. All patients had CV risk factors at baseline. Of note, the total cholesterol, and both LDL and HDL cholesterol fractions significantly increased during treatment. On the contrary, triglycerides concentrations had not significant variations, and neither a significant increase of HbA1c was observed.

Table 6. Cardiovascular adverse events

<table>
<thead>
<tr>
<th>Overall incidence</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery disease</td>
<td>3</td>
</tr>
<tr>
<td>Arterial thrombosis</td>
<td>2</td>
</tr>
<tr>
<td>QTc prolongation</td>
<td>1</td>
</tr>
<tr>
<td>Atrioventricular block</td>
<td>1</td>
</tr>
<tr>
<td>Overall incidence</td>
<td>7/130 (5%)</td>
</tr>
</tbody>
</table>

4.1.6. Patient disposition

After a median follow-up of 24 months (range 18-36) all the enrolled patients are alive, and 107 (82%) of the patients are still on study (Table 7). The nilotinib daily dose was 800 mg, 600 mg, 300 mg or less, in 2%, 75%, and 5% of the patients, respectively. The main reason for treatment discontinuation was toxicity (11 patients, 8%); other reasons included failures (5 patients, 4%), and progression to accelerated/blast phase (2 patients, 2%).

Table 7. Patient disposition

<table>
<thead>
<tr>
<th></th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Still on study</td>
<td>107 (82)</td>
</tr>
<tr>
<td>Off-study</td>
<td>23 (18)</td>
</tr>
<tr>
<td>Progression to AP/BP</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Failure</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Toxicity</td>
<td>11 (8)</td>
</tr>
<tr>
<td>Other*</td>
<td>5 (4)</td>
</tr>
</tbody>
</table>

* Pregnancy, lost follow-up, withdrawal of informed consent
4.2. Biological results

4.2.1 Samples

CD34+ cells were purified by immune-magnetic separation from peripheral blood (PB) of 7 newly diagnosed chronic phase (CP) CML patients and compared to the normal counterpart obtained from normal bone marrow of three healthy donors (NBM) and/or from umbilical cord blood (CB) of three donors. CP-CML samples were obtained from patients with WBC counts ranging between 41,900 to 421,400; Sokal score resulted intermediate in six patients and low in one (Table 8).

**Table 8. Characteristics of patients with CD34+ cells collected at diagnosis**

<table>
<thead>
<tr>
<th>#</th>
<th>Source</th>
<th>Disease Phase</th>
<th>Sokal score</th>
<th>Transcript type</th>
<th>WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB</td>
<td>CP</td>
<td>INT</td>
<td>b2a2</td>
<td>135400</td>
</tr>
<tr>
<td>2</td>
<td>PB</td>
<td>CP</td>
<td>LOW</td>
<td>b3a2</td>
<td>98300</td>
</tr>
<tr>
<td>3</td>
<td>PB</td>
<td>CP</td>
<td>INT</td>
<td>b3a2</td>
<td>54000</td>
</tr>
<tr>
<td>4</td>
<td>PB</td>
<td>CP</td>
<td>INT</td>
<td>b3a2</td>
<td>62000</td>
</tr>
<tr>
<td>5</td>
<td>PB</td>
<td>CP</td>
<td>INT</td>
<td>b3a2</td>
<td>421400</td>
</tr>
<tr>
<td>6</td>
<td>PB</td>
<td>CP</td>
<td>INT</td>
<td>b2a2/b3a2</td>
<td>97500</td>
</tr>
</tbody>
</table>

4.2.2. Analysis of the phospho-proteomic profile

The comparison between the phospho-proteomic profile of CP-CML CD34+ cells and NBM CD34+ cells (Figure 7, 8) showed that the former are characterized by:

1) lower phosphorylation of STAT2 (p=0.023), STAT5 (p=0.036), and of tyrosine kinases of the Src family - Lck, Fyn, Lyn, and Yes (p=0.04) -, involved in the regulation of growth and cell survival;

2) higher phosphorylation of p53, at Ser15 (p=0.047).
Figure 7. Analysis of phospho-proteomic profile reveals that STATs and SRK family members resulted differently activated in CML CD34+ cells

<table>
<thead>
<tr>
<th>MAPKs family</th>
<th>PI3K family</th>
<th>STATs family</th>
<th>p53</th>
<th>Tyrosine kinases</th>
<th>Cell Cycle Inhibitors</th>
<th>Molecular chaperones</th>
<th>Cell growth, adhesion and migration</th>
<th>Homeostasis sensors</th>
<th>Ion (Ca2+) channel regulator</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38α (T180/Y182) ERK1/2 (T202/Y204, T185/Y187) RSK1/2/3 (S380/S386/S377)</td>
<td>AKT (S473) AKT (T308) mTOR (S2448)</td>
<td>STAT2 (Y689) STAT3 (Y705) STAT5α (Y694) STAT5β (Y699)</td>
<td>p53 (S392) p53 (S466) p53 (S15)</td>
<td>GSK-3α/β (S21/S99) Src (Y419) Lyn (Y397) Lck (Y394) Fyn (Y420) Yes (426) Fgr (Y412) Hck (Y411) Chk-2 (T68) FAK (Y397)</td>
<td>p27 (T198) HSP27 (S78/S82) β-Catenin</td>
<td>AMPKβ1 (T174) AMPKβ2 (T172) eNOS (S1177)</td>
<td>Pyk2 (Y402)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2.3 Analysis of the expression of apoptotic proteins

The analysis of the expression of 32 apoptotic proteins revealed that CD34+ cells from CP-CML, compared to normal CD34+ cells (Figure 9, 10), are characterized by:

1) lower expression of the catalase (p=0.044), an enzyme that protects cells from the toxic effects of hydrogen peroxide and promotes growth of normal and neoplastic cells including myeloid leukemia cells;

2) higher expression of FADD, a death receptor involved in extrinsic apoptosis and necroptosis, and of the heat shock proteins HSP60 and HSP70, essentials for the survival of the cells after toxic stimuli.
Figure 9. FADD, HSP60, HSP70 and catalase are differently expressed in CML CD34+ cells compared to normal CD34+ cells.

Bcl-2 family
- Bad
- Bax
- Bcl-2
- Bcl-x

Death receptors
- TRAIL R1/DR4
- TRAIL R2/DR5
- FADD
- Fas/TNFRSF6
- TNF R1/TNFRSF1A

Caspases
- Pro-Caspase-3
- Cleaved-Caspase-3

Caspase inhibitors
- cIAP-1
- cIAP-2
- XIAP
- Livin

Apoptosis inhibitors
- Survivin

Apoptosis activators
- Cytochrome c
- SMAC/Diablo
- HIF-1α

Molecular chaperones
- HO-1/HMox1/Hsp32
- HO-2/HMox2
- Hsp27
- Hsp60
- Hsp70
- HTRA2/Omi
- Clusterin

ROS scavenging
- Catalase
- PON2

Cell Cycle Inhibitors
- p21/CIP1/CDKN1A
- p27/Kip1

Cellular checkpoint
- Claspin
- phospho-Rad17 (S635)

Densitometry (Ab/ref)

* = p<0.05
Figure 10. Detail of HSP70 expression

- nBM CD34+ cells
- CML CD34+ cells

HSP70

<table>
<thead>
<tr>
<th>Density (Ab/ref)</th>
<th>CML CD34+ cells</th>
<th>nBM CD34+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( p = 0.003 \)
5. Discussion

Nilotinib is a derivative of imatinib with greater potency and specificity against the tyrosine kinase activity of BCR-ABL1, the characteristic leukemogenic protein of chronic myeloid leukemia (CML)\textsuperscript{160}. Due to the efficacy shown in imatinib resistant or imatinib-intolerant patients, nilotinib has been tested in the first-line setting. The randomized phase III trial ENESTnd demonstrated the superior efficacy of nilotinib (both at 300 mg and 400 mg twice daily [TD]) compared to imatinib 400 mg once daily\textsuperscript{25}. On the basis of this study, Nilotinib was approved for the first-line treatment of CML at the dose of 300 mg TD (less toxic and equally effective). The latest update, with 5 years of follow-up, confirmed the superior anti-leukemic activity of nilotinib, with higher progression-free survival, and higher rates of deep molecular responses\textsuperscript{161}. Only other two small phase II trials testing prospectively nilotinib frontline (400 mg TD) have been so far published by independent investigators (GIMEMA and the MD Anderson)\textsuperscript{23,149}. These studies, although with a short follow-up (median less than 18 months)\textsuperscript{23,149}, showed that nilotinib induced high and fast response rates, with few progressions to accelerated-blast phase (AP/BP), and favorable early toxicity profile. On the other hand, nilotinib-associated atherosclerotic adverse events (AAE) were recently uncovered, particularly peripheral arterial occlusive disease\textsuperscript{28,30,31,150,151}, raising concerns on the long-term safety of this drug. Since it may be possible that nilotinib will be more and more used for the 1st line treatment of CML, this prospective phase III-b trial was performed by the GIMEMA CML WP to explore and to assess the efficacy and safety of nilotinib in a multicenter, independent study of an unselected cohort of patients. Here, after a median follow-up of 24 months, all patients are alive, and only 2 patients have progressed to AP/BP. Therefore, our study confirmed that nilotinib is highly effective in the prevention of the progression to AP/BP, a condition that today is still associated with high mortality rates. Failures according to ELN 2013 recommendations were few (7 patients, 5.3%); however, about a quarter of patients have discontinued nilotinib treatment by 24 months, being toxicity the most common reason (11 patients, 8.4%). Despite the
relatively short follow-up, cardiovascular issues, particularly atherosclerotic adverse events (AE), have emerged. The frequency of these AEs may counterbalance the anti-leukemic efficacy. Therefore, it seems crucial to identify at diagnosis patients at higher risk for atherosclerotic complications during nilotinib therapy, evaluating baseline known CV risk factors, promoting the improvement of modifiable ones, and, possibly, as recently described\textsuperscript{162}, studying biochemical and genetic traits predictive of thrombotic vascular events. Importantly, for patients with an unfavorable CV risk profile, other TKIs (i.e. imatinib, dasatinib) may be used.

Currently, one of the most important targets of the treatment of CML with TKIs is the achievement of the deep MR, marker of better long-term outcome. Moreover, deep MR has been identified as a prerequisite to treatment discontinuation\textsuperscript{156,163}. The primary endpoint of the present study is the rate of MR 4.0 at 24 months, which can provide important information to plan and perform future studies aimed at treatment discontinuation, either primarily or after addition of other drugs targeting residual leukemic stem cells. Here, the estimated 24-month rates of MR 4.0 and MR 4.5 were 47% and 22%, respectively. These results compare favorably to those obtained with imatinib, in historic cohorts, and confirm the findings of the Company-sponsored ENESTnd study\textsuperscript{164}.

Additional important information derived from the present trial, and not yet reported in other nilotinib studies, is the stability of the deep molecular response. A stable molecular response for one year or more was maintained by 27/57 (47%, or 21% of the total) patients that achieved a MR 4.0, and by 6/30 (20%, or 5% of the total) patients that obtained a MR 4.5, respectively. Considering the increasing rates of deep MR over time (MR 4.0: 2%, 12%, 28%, 29% at 3, 6, 12, 18 months, respectively; MR 4.5: 0%, 2%, 7%, 12% at 3, 6, 12, 18 months, respectively), a significant proportion of patients will be candidate to treatment discontinuation in the next years, with higher probability of remaining disease-free in the long term. The advantages of this possible future scenario could be: first, the possibility of treatment discontinuation, at least in patients with chronic clinical adverse events; second, a potential reduction of the costs of TKI
treatment (after the introduction of TKI, the costs of CML treatment is increasing year by year, with the increasing prevalence of CML patients).

However, even when a stable deep MR is reached, a significant proportion of patients, 40 – 60% depending to the molecular criteria used, will eventually relapse\textsuperscript{156,163}. In almost all cases the restarting of the treatment with TKI was able to restore a molecular response.

The persistence of detectable disease in most patients, and the clinical relapses observed after TKI discontinuation despite a prolonged deep molecular response, are mainly related to the persistence of a quiescent population of leukemic stem cells. Thus, targeting the leukemic stem cells could be necessary for the eradication of the disease. Several mechanisms are implicated in leukemic stem cells persistence, and probably most of them are similar to those active in normal stem cells. Therefore, the characterization of the leukemic stem cells and the understanding of their differences compared to the normal counterpart are of utmost importance.

In our study, patient's samples were collected at the CML diagnosis, prior to any anti-leukemic treatment, to separate the CD34+ stem cells. We focused on their proteomic characterization in comparison to CD34+ cells derived from healthy donors. The results showed a different phospho-proteomic profile between the leukemic and normal CD34+ cells. In particular, in the CD34+ CML cells we found: a lower phosphorylation of STAT2, STAT5, and tyrosine kinases of the Src family (Lck, Fyn, Lyn, and Yes), involved in the regulation of growth and cell survival; a higher phosphorylation of p53.

The analysis of the expression of proteins involved in the apoptotic machinery and in the cell cycle regulation, revealed that CD34+ cells from CP-CML, are characterized by lower expression of the catalase, an enzyme that protects cells from the toxic effects of hydrogen peroxide and promotes growth of normal and neoplastic cells; a higher expression of FADD, a death receptor involved in extrinsic apoptosis and necroptosis, and of the heat shock proteins HSP 60 and HSP70, essentials for the survival of the cells after various cytotoxic stimuli.
Taken together, these data show that CD34+ cells from leukemic patients at diagnosis, have a proteomic profile that promotes the quiescence through the inhibition of proliferation, and that favors cell survival despite cytotoxic stimuli. These characteristics may explain, at least partially, why these cells are resistant to the treatment with TKIs, which is highly effective in targeting more differentiated and proliferating cells, and, ultimately, why these CD34+ cells can determine the relapse after treatment discontinuation.

The presence of these additional and complex changes in the signaling network of chronic phase CML must be taken into account for the investigation on novel targeted therapies, aimed at the eradication of the disease.
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