HIGH BCR-ABL/GUS^{IS} LEVELS AT DIAGNOSIS OF CHRONIC PHASE CML ARE ASSOCIATED WITH UNFAVORABLE RESPONSES TO STANDARD-DOSE IMATINIB

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Running Title: High BCR-ABL at diagnosis induces poor imatinib responses

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Translational Relevance: Our results show that subjects expressing high *BCR-ABL/GUS*^{IS} transcripts at diagnosis are less likely to achieve optimal responses according to the current ELN criteria if treated with Imatinib 400 mg daily. Applying ROC curves for selected survival outcomes it is also possible to determine specific *BCR-ABL/GUS*^{IS} thresholds that identify patients (displaying *BCR-ABL/GUS*^{IS} transcripts above these thresholds) showing significantly inferior rates of failure-free, event-free and transformation-free survival. Elevated *BCR-ABL/GUS*^{IS} transcripts at diagnosis are as reliable as the 10% *BCR-ABL/ABL*^{IS} limit after 3 months and the 1% *BCR-ABL/ABL*^{IS} threshold after 6 months of therapy. Finally, patients displaying high *BCR-ABL/GUS*^{IS} transcripts at diagnosis are likely to present >10% *BCR-ABL/ABL*^{IS} values after 3 months of treatment and >1% *BCR-ABL/ABL*^{IS} after 6 months of therapy. In summary, our data suggest that high *BCR-ABL* transcripts measured at diagnosis employing *GUS* as a reference

gene identify CML patients unlikely to benefit from standard dose imatinib.

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ABSTRACT

Purpose: The approval of second-generation tyrosine kinase inhibitors (TKIs) for the first line treatment of chronic myeloid leukemia (CML) has generated an unmet need for baseline molecular parameters associated with inadequate imatinib responses.

Experimental Design: We correlated *BCR-ABL/GUS*^{IS} and *BCR-ABL/ABL* transcripts at diagnosis with the outcome - defined by the 2013 European LeukemiaNet recommendations - of 272 newly diagnosed CML patients receiving Imatinib 400 mg/daily. Applying Receiver Operating Characteristic curves we defined *BCR-ABL/GUS*^{IS} and *BCR-ABL/ABL* levels associated with lower probabilities of optimal response, failure-free (FFS), event-free (EFS), transformation-free (TFS) and overall survival (OS).

Results: With a median follow-up of 60 months, 65.4% of patients achieved an optimal response (OR), 5.6% were classified as "warnings", 22.4% failed imatinib and 6.6% switched to a different TKI because of drug intolerance. We recorded 19 deaths (6.9%), 7 (2.5%) attributable to disease progression. We found that higher *BCR-ABL/GUS*^{IS} levels at diagnosis were associated with inferior rates of OR ($p<0.001$), FFS ($p<0.001$) and EFS ($p<0.001$). Elevated *BCR-ABL/GUS*^{IS} levels were also associated with lower rates of TFS ($p=0.029$) but not with OS ($p=0.132$). Similarly, high *BCR-ABL/ABL* levels at diagnosis were associated with inferior rates of OR ($p=0.03$), FFS ($p=0.001$) and EFS ($p=0.005$), but not with TFS ($p=0.167$) or OS ($p=0.052$). However, in internal validation experiments, *GUS* outperformed *ABL* in samples collected at diagnosis as the latter produced 80% misclassification rates.

Conclusions: Our data suggest that high *BCR-ABL* transcripts at diagnosis measured employing *GUS* as a reference gene identify CML patients unlikely to benefit from standard dose imatinib.

Introduction

The BCR-ABL oncoprotein is the culprit of chronic myeloid leukemia (CML) as it transforms the hematopoietic stem cell by altering its proliferation rate, survival signaling, cytoskeleton dynamics and immunological interactions (1-5). Imatinib mesylate (IM) has dramatically improved the outcome of CML patients in chronic phase (CP), generating unprecedented rates of complete hematologic (CHR) and cytogenetic (CCyR) responses and sustained reductions in *BCR-ABL* transcripts (6-9). Despite these results, approximately 50% of CML patients fail to achieve an optimal response as defined by the current European Leukemia Net (ELN) recommendations. Intolerance, suboptimal responses and the emergence of drug failure all contribute to identify a group of patients that do not benefit from IM (10,11). Interestingly, assessing disease risk at diagnosis with the Sokal and Hasford scores has maintained its clinical significance as patients classified as high-risk are less likely to attain the desired cytogenetic and molecular responses (6,12,13).

The search for accurate baseline parameters associated with unsatisfactory outcomes has led a group of CML investigators to devise the EUTOS score that assigns patients to low/high-risk categories according to spleen size and basophil count. High-risk subjects display inferior rates of CCyR, progression-free (PFS) and overall survival (OS) (14,15). However, the original EUTOS score was developed to predict the probability of obtaining a CCyR within 18 months. Hence, in a later effort, the same investigators have proposed a EUTOS Long-Term Survival (ELTS) score that employs age, spleen size, blast number and platelet counts to subdivide CML patients in low-, intermediate- or high-risk groups (16). Subjects assigned to the latter two groups display a significantly higher risk of dying from CML progression (17).

However, while each of these scores has been validated in multiple patient series, none of them is specifically associated with response to IM. In this study, we searched for an easily detectable molecular parameter that would identify, at diagnosis, CML patients unlikely to benefit from IM. This issue has become of pivotal importance with the approval of second generation (2G) tyrosine kinase inhibitors (TKIs) for the first line treatment of the disease (8,18,19). Indeed, considering the excellent results achieved with IM, the availability of generic forms of the drug at highly reduced costs, and the shorter follow-up of the studies employing 2G TKIs in first line, a molecular indicator associated with inadequate IM responses could distinguish patients that will benefit from the drug from those that would require alternative treatments (20-26). We report that high *BCR-ABL/GUS*^{IS} transcripts measured at baseline are suggestive of inferior probabilities of achieving optimal responses to standard dose (400 mg/daily) IM.

Materials and Methods

Patient Characteristics and Treatment

Between January 1, 2006 and December 31, 2015, 272 unselected adult patients with CML in CP were accrued to the observational SCREEN (Sicily and Calabria CML REgional ENterprise) multi-center study and analyzed for clinical, cytogenetic and molecular responses. The study is on going but data collection was limited to the first 272 individuals that presented ≥12 months of follow-up. The research ethics committee of each recruiting institution reviewed and approved the study protocol and all patients gave written informed consent. IM - 400 mg/daily - was started within twelve weeks from diagnosis and discontinued in the presence of grade 3/4 toxicities. Treatment was resumed after toxicity reduction to grade 1 or after complete resolution. IM responses were evaluated according to the 2013 ELN criteria (27).

Hematologic and Cytogenetic Responses

CP-CML and CHR were defined by conventional criteria (28). Bone marrow (BM) cytogenetics were assessed at diagnosis and then every 6 months until achievement of a CCyR. Subsequently, annual BM examinations were performed although, after 2010, subjects in CCyR were mostly monitored by real-time quantitative polymerase chain reaction (RQ-PCR). Cytogenetic responses were evaluated on ≥20 marrow cell metaphases. CCyR was defined as failure to detect Philadelphia chromosome (Ph)-positive metaphases in two consecutive examinations. Confirmed detection of Ph-positive metaphases after acquiring CCyR was considered cytogenetic relapse. Chromosomal abnormalities were scored as previously described (29). When cytogenetic analyses were unavailable due to insufficient material, technical failure or patient's refusal to undergo a BM

aspirate, *BCR-ABL/ABL*^{IS} ratios ≤1% were considered equivalent to a CCyR as previously reported (30).

Quantification of BCR-ABL Transcripts

BCR-ABL transcripts were measured from peripheral blood (PB) samples drawn at diagnosis and every three months thereafter using RQ-PCR (31). All RQ-PCR determinations were centralized in the Center of Experimental Oncology and Hematology. Samples collected at diagnosis were subjected to RQ-PCR employing the TaqMan platform and both beta-glucuronidase *(24)* and *ABL* as reference genes as previously reported (32). For samples collected at diagnosis we used *GUS*, as it is the more appropriate reference gene for specimens expressing high levels of *BCR-ABL* (32). For *BCR-ABL* transcripts measured at time-points other than diagnosis, *ABL* was the only reference gene employed (31,33). *BCR-ABL*/*GUS* and *BCR-ABL*/*ABL* ratios were reported on the international scale (IS) using a conversion factor (CF) calculated - on a yearly basis - from primary CML samples shared with the laboratory at the University Hospital Mannheim. When BCR-ABL/ABL was >10% and a specific value was reported, the suffix IS was removed as according to previously published data (34) the IS should not be calculated for BCR-ABL/ABL ratios >10% based on non-linearity of the ratio. RQ-PCR determinations were considered of appropriate quality only in the presence of no less than 24.000 *GUS* copies or 10.000 *ABL* copies as previously indicated (35). Of the 272 PB specimens collected, 32 had to be discarded because of prior HU exposure (n=27) or poor nucleic acid quality (n=5). Of the 240 remaining specimens, only 2 had *BCR-ABL/ABL*^{IS} ratios <10%. Unavailable baseline samples were distributed as follows: 17 of 178 specimens from patients achieving an OR, 7 of 61 samples from patients failing IM, 3 of 15 specimens from individuals classified as “warning” and 5 of 18 samples from intolerant patients. Two hundred-five and 214 blood samples - respectively - were available for molecular analyses performed at the 3 and 6

month time points (Table 3). For analyses requiring both baseline and 3-month samples, blood specimens were available from 200 patients. Similarly, blood samples from 210 patients were assessable at diagnosis and after 6 months of IM (Table 4). Major Molecular Response and MR^4 have been defined as previously reported (36).

Statistical Analyses

Univariate probabilities of overall survival (OS), transformation-free (TFS), failure-free (FFS) and event-free (EFS) survival were calculated using the Kaplan-Meier method. Statistical significance of Kaplan-Meier curve differences was evaluated using the Mantel-Haenszel test as previously described (37). TFS was defined as survival without disease progression to accelerated or blast phases. FFS was defined as survival without evidence of drug failure according to the current ELN criteria. Events included in our definition of EFS were: death from any cause, progression from chronic phase, IM failure according to the 2013 ELN recommendations and development of intolerance. Probabilities of cytogenetic and molecular responses were calculated using cumulative incidence function within the Kaplan-Meier method, in which maintained responses were the events of interest. Significance of *BCR-ABL* transcript levels at diagnosis for the achievement of OS, TFS, FFS, EFS and OR were evaluated using the Wilcoxon-rank-sum test. Optimal threshold in $BCR\text{-}ABL/GUS^{IS}$ levels associated with specific outcomes after IM were calculated using Receiver Operating Characteristic (ROC). These thresholds maximize true positive and false positive rates in a binary classification according to the clinical phenotype of interest. P values were two-sided with 95% confidence intervals (CI). Analyses were performed using the R software (38). To assess the robustness of the association between $BCR\text{-}ABL^{IS}$ levels at diagnosis and FFS, the database was randomly shuffled and divided into a training set (with 80% of patients) and a test set (with 20% of patients) using a simple perl script. The sampling procedure was

iterated in order to select more than 50 combinations of randomly selected pairs of training and test subsets with a comparable distribution of patients with *BCR-ABL* levels above and below the specified threshold.

Results

Treatment Response, OS, TFS, ELN Outcomes and second line therapies

Patient characteristics are summarized in Supplementary Table 1. All patients achieved a CHR. 229 patients (84.2%) attained a CCyR (median time 6 months; range 6-24 months), 206 (75.7%) obtained a MMR (median time 12 months; range 3-57 months), and 68 (25%) displayed an MR^4 (median time 34.5 months; range 6-57 months). Median follow-up of the accrued population was 60 months (range, 12-108 months). No patients were lost to follow-up. Eight-year estimates of OS (89%) and TFS (95%) on an intention-to-treat basis are shown in Supplementary Figure 1. Comparison with five- and eight-year results reported for the IRIS (39) and German CML IV studies (40,41) are summarized in Supplementary Table 2. Using the 2013 ELN recommendations, at the time of data lock 178 patients (65.4%) displayed an OR, 61 (22.4%) had failed IM, 15 (5.6%) were classified as "warning" and 18 (6.6%) discontinued IM due to intolerance. Of the 61 patients failing treatment, 1 underwent allogeneic stem-cell transplantation while the remaining 60 received 2G TKIs. Twenty-three of the 60 patients that eventually switched to dasatinib or nilotinib transiently received increased (600/800 mg/daily) IM doses for a median of 13 months (range 2-39). All 15 intolerant patients received 2G TKIs.

Correlation between BCR-ABL/GUS^{IS} or BCR-ABL/ABL transcripts at baseline and IM response

Median *BCR-ABL/GUS*^{IS} levels at diagnosis in the entire population were 13.6% (range 2.1-66.7%), while median *BCR-ABL/ABL* expression was 67.2% (range 5.6-349.5%). To establish if quantification of *BCR-ABL* transcripts at diagnosis would correlate with IM response, we stratified our evaluable patient cohort in optimal responders (n=161, 178 minus 17 unavailable samples as described in Methods) and individuals failing IM (n=54, 61 minus 7 unavailable samples), and we

then analyzed the amount of *BCR-ABL/GUS*^{IS} expression in each group. Subjects failing IM presented significantly higher amounts of *BCR-ABL* transcripts when compared to optimal responders (20.39% vs 11.97%; p <0.001; Figure 1). We then repeated the same analysis employing *ABL* as a control gene and again found that patients failing IM displayed higher *BCR-ABL* transcripts when compared to optimal responders (101.69% vs 61.35%; p <0.005; Supplementary Figure 2). While the low number of evaluable "warning" (n=12, 15 minus 3 unavailable samples) or intolerant (n=13, 18 minus 5 unavailable samples) patients did not allow any meaningful statistical analyses, median baseline *BCR-ABL/GUS* and *BCR-ABL/ABL* levels observed in these groups were similar to those detected in optimal responders.

Definition of BCR-ABL/GUS^{IS} and BCR-ABL/ABL thresholds correlated with IM response

We next wanted to determine individual *BCR-ABL/GUS*^{IS} *and BCR-ABL/ABL* thresholds associated with specific outcomes after IM treatment. We employed ROC curves to define baseline *BCR-ABL* values that would correlate with lower rates of OS, TFS, FFS, and EFS and with lower probabilities of OR (Table 1 and Supplementary Table 3). While we identified a *BCR-ABL/GUS*^{IS} threshold for OS (18.55%), this value did not achieve statistical significance (p=0.132) (Figure 2A). However, we found that *BCR-ABL/GUS*^{IS} levels >18.79% and >14.89% were associated with a lower likelihood of achieving a TFS (p=0.029) and FFS (p<0.001), respectively (Figure 2B, C). Likewise, we observed that a *BCR-ABL/GUS*^{IS} value >15.94% discriminated patients with significantly lower probabilities (p<0.001) of obtaining both an EFS and an OR (Figure 2D and Table 1). When we employed *BCR-ABL/ABL* transcripts to define outcome-related thresholds, the values calculated for OS (45.07%) and for TFS (44.50%) failed to achieve statistical significance (p=0.052 and p=0.167, respectively) (Supplementary Table 3 and Supplementary Figure 3A, B). On the contrary, *BCR-ABL/ABL* levels >92.07%, >102.41% and >97.36% were significantly associated with lower probabilities of FFS

(p=0.001), EFS (p=0.005) and OR (p<0.03; Supplementary Table 3 and Supplementary Figure 3C, D).

Performance of the models based on BCR-ABL/GUS^{IS} or BCR-ABL/ABL thresholds associated with IM response

We next wanted to validate the performance of the *GUS*-based and the *ABL*-based models in accurately discriminating patients unlikely to achieve specified treatment outcomes with standard dose IM. We chose an internal validation method that employs repeated unbalanced splits to subdivide the overall population in training and validation sets (42,43). The principle behind this approach is that by randomizing patients according to the same normalization parameter using numerically unbalanced splits (80/20), the smaller subgroup will highlight possible inconsistencies, which may be masked in the general population of the study. We therefore randomized our 272 patients for 50 times in different 80/20 groups employing the FFS ROC values calculated using either *GUS* (Table 1) or *ABL* (Supplementary Table 3) as reference genes. We found that *GUS* clearly outperformed *ABL* as a control gene for samples measured at diagnosis (Table 2). Indeed, GUS appropriately classified patients at risk of failing IM in 100% of the 80% splits (training set) and in 96% of the 20% splits (validation set). On the contrary, ABL was reliable in the 80% splits (training set) appropriately classifying 94% of the patients, but generated an unacceptable 80% misclassification rate (20% correct classification) in the smaller 20% splits (validation set). These findings confirm that, unlike *GUS, ABL* cannot be used as a reference gene for samples collected at diagnosis.

Comparison of different molecular parameters associated with IM response

Consolidated evidence has established that *BCR-ABL/ABL*^{IS} values >10% after three months and >1% after six months of TKI therapy are associated with inferior rates of OS, progression-free survival (PFS), EFS and CCyR (44,45). We wished to compare these two molecular parameters with the different *BCR-ABL/GUS*^{IS} thresholds that we had previously identified as, unlike *BCR-ABL/ABL* transcripts, *BCR-ABL/GUS*^{IS} levels can be reliably measured before commencing TKI treatment and may therefore guide the choice of the most appropriate first-line drug (32). We found that baseline *BCR-ABL/GUS*^{IS} thresholds for OR, EFS and FFS were equally effective in predicting the probability of achieving these outcomes as compared to the 10% or 1% *BCR-ABL/ABL*^{IS} cut-offs ($p<0.001$; Table 3). In our cohort, only the 18.79% *BCR-ABL/GUS*^{IS} threshold at diagnosis ($p=0.029$) and the 1% *BCR-ABL/ABL*^{IS} value after 6 months of treatment ($p=0.021$) could significantly discriminate the likelihood of obtaining a TFS (Table 3). This was not the case for the 10% *BCR-ABL/ABL*^{IS} level after 3 months of therapy ($p=0.134$). As for OS, only the 1% *BCR-ABL/ABL*^{IS} threshold after 6 months of IM correlated with higher survival rates ($p=0.025$). Neither the baseline 18.55% *BCR-ABL/GUS*^{IS} value ($p=0.132$) nor the 10% *BCR-ABL/ABL*^{IS} cut-off after 3 months of therapy ($p=0.132$) were associated with higher survival probabilities (Table 3).

Co-classification of CML patients by different molecular parameters

We next wanted to establish whether patients with high *BCR-ABL/GUS*^{IS} transcripts at diagnosis would also display higher *BCR-ABL/ABL*^{IS} values after three or six months of IM. We compared the populations defined by *BCR-ABL/GUS*^{IS} thresholds calculated for each outcome with those identified by the 10% (at 3 months) and 1% (at 6 months) *BCR-ABL/ABL*^{IS} levels. Our analysis showed high concordance between the three molecular parameters (Table 4). Specifically, patients failing to achieve an OR (>15.94% *BCR-ABL/GUS*^{IS}) or exhibiting lower rates of EFS (>15.94% *BCR-ABL/GUS*^{IS}), FFS (>14.89% *BCR-ABL/GUS*^{IS}), TFS (>18.79% *BCR-ABL/GUS*^{IS}) and OS

(>18.55% *BCR-ABL/GUS*^{IS}) mostly displayed *BCR-ABL/ABL*^{IS} levels >10% at 3 months and >1% at 6 months with concordance ranging from 61.5% to 72.3% (Table 4). All these concordance rates were statistically significant.

Discussion

In this first account of the SCREEN study, we describe the correlation between *BCR-ABL/GUS*^{IS} levels measured at diagnosis and the response to IM of 272 newly diagnosed CP-CML patients. Main aim of the study was to determine if the quantitative evaluation of *BCR-ABL* transcripts before exposure to any therapy could identify patients unlikely to benefit from standard-dose IM. In our analysis, higher levels of *BCR-ABL* expression were associated with unsatisfactory responses to the drug.

These findings pose several interesting questions. First of all, our data are apparently in contrast with what has been previously published by Hanfstein and colleagues on behalf of the German CML Study Group (46). In their report, the authors investigated the possible predictive value of *BCR-ABL* transcripts measured - at diagnosis - using *GUS* as a reference gene in a subgroup of 301 patients from the IM-treated CML cohort recruited to the German CML Study IV. Authors correctly excluded from their analysis samples collected after exposure to HU or IM. They found no correlation between high *BCR-ABL* expression and OS and PFS and therefore argued for the limited value of quantifying *BCR-ABL* per se at diagnosis. Just like Hanfstein et al. we failed to detect any significant correlation between baseline *BCR-ABL/GUS*^{IS} levels and OS or PFS, respectively (Table 1 and data not shown). However, a careful analysis of our results suggests that this is due to the limited number of events detected in our patient cohort. Specifically, of the 19 deaths observed - to date - in our patient population, only 7 were due to disease progression. Hence, most of these events were probably unrelated to CML. This explains why high *BCR-ABL/GUS*^{IS} expression was associated with TFS but not with PFS (that includes deaths from any cause in addition to those due to disease transformation) and also explains why the *BCR-ABL/GUS*^{IS} threshold calculated for OS

(18.55%) was inferior to that found for TFS (18.79%). It should also be noted that, while overall efficacy of IM-based treatments in the German CML Study IV were superimposable to those observed in our series with standard dose IM (Supplementary Table 2), there were significant differences in the baseline *BCR-ABL/GUS*^{IS} quantification of the two patient cohorts. Indeed, both median *BCR-ABL/GUS*^{IS} expression (13.6% in the SCREEN study vs 33% in the German CML Study IV) and the observed *BCR-ABL/GUS*^{IS} ranges (2.1%-66.7% in our series vs 0.1%-230% in the German cohort) argue for a higher degree of heterogeneity and, possibly, for more patients with unfavorable disease characteristics in the German population. It should also be noted that - unlike our cohort - only 75 (25%) of the 301 patients analyzed by the German group received IM 400 mg daily. This observation is of critical importance as our preliminary data suggest that employing higher IM doses or more potent 2G TKIs raises the thresholds identified in this study for standard dose IM. Finally, the lack of correlation that we report between the 10% *BCR-ABL/ABL*^{IS} threshold and TFS and OS rates (Table 2) should not be surprising since the German group observed the same result when they limited their analysis to a relatively small (i.e. 301 subjects) patient cohort (46).

Another critical issue concerns possible correlations between baseline *BCR-ABL/GUS*^{IS} levels and different clinical and molecular parameters. In a univariate analysis, we found no associations between *BCR-ABL/GUS*^{IS} transcripts at diagnosis and patient age, sex, hemoglobin levels, Sokal risk scores, number of Ph-positive metaphases and white blood cell (WBC) counts. Interestingly, the lack of correlation between WBC counts at diagnosis and *BCR-ABL/GUS*^{IS} levels suggests that high *BCR-ABL* values are independent from the leukemic burden (i.e. from the number of Ph-positive cells at diagnosis). For example, in our series the patient with the highest WBC count at diagnosis (patient #25: WBCs 758.000/µL) expressed low (5.51%) *BCR-ABL/GUS*^{IS}, while the individual (#157)

with the highest *BCR-ABL/GUS*^{IS} value at diagnosis (66.71%) only had 50.000/ µL WBCs. Thus, high *BCR-ABL/GUS*^{IS} expression is probably indicative of higher amounts of *BCR-ABL* transcripts within each leukemic cell, a well-established sign of CML disease progression (47). An additional observation strengthens this hypothesis: the median baseline *BCR-ABL/GUS*^{IS} levels of the 7 patients deceased after CML progression was 33.85% (range 23.09%-41.23%), a much higher value than the 20.39% displayed by the 61 patients failing IM.

From a technical standpoint, our results provide further evidence supporting the concept that *ABL* is not an appropriate reference gene for samples presenting high *BCR-ABL* transcripts (i.e. >10% *BCR-ABL/ABL*^{IS}) as these specimens require a *BCR-ABL*-indipendent housekeeping gene. Indeed, internal validation experiments in our patient cohort clearly showed that *ABL* may missclassify up to 80% of patients at risk of failing IM (Table 2). The importance of employing the correct reference gene when measuring *BCR-ABL* expression at diagnosis is highlighted by the data summarized in Supplementary Figure 4. Both patients depicted in the upper panel presented high *BCR-ABL/GUS*^{IS} values at baseline (54.9% and 40.2%) but low *BCR-ABL/ABL* transcripts (33.8% and 15.8%), yet both subjects failed IM treatment after 6 and 12 months, respectively. It will ultimately be up to each individual laboratory to choose between using different reference genes for samples collected at diagnosis (24) or at other time points (*ABL*) or alternatively switching to *GUS* for all their molecular determinations.

From a biological standpoint, our findings raise several - yet unanswered - issues. Our data suggest that the fate of the leukemic clone may be already determined at the time of diagnosis. However, it is still unclear if quantitative or qualitative mechanisms underlie the inferior IM sensitivity of CML clones with high *BCR-ABL.* It is likely, although untested, that higher *BCR-ABL* transcripts

translate to higher expression of the BCR-ABL oncoprotein and increased tyrosine kinase activity. According to the quantitative hypothesis, this would ultimately strengthen canonical BCR-ABL-dependent signaling resulting in a less responsive leukemic population. On the other hand, the qualitative hypothesis assumes that higher *BCR-ABL* activity leads to "leakage" of BCR-ABL signaling to downstream targets that are usually not involved in BCR-ABL-dependent transformation. It is also possible that both quantitative and qualitative mechanisms contribute to the lower IM responsiveness of patients displaying high levels of *BCR-ABL*. It also remains to be seen if CD34+ progenitors derived from patients presenting high *BCR-ABL/GUS*^{IS} levels at diagnosis display higher *BCR-ABL* transcripts as compared to CD34+ cells isolated from patients with low *BCR-ABL/GUS*^{IS}.

From a clinical standpoint, additional studies will be needed to compare the predictive potential of the ELTS with that of *BCR-ABL/GUS*^{IS} quantification at diagnosis. Similarly, the German and the Australian groups have suggested that dynamic measurements of *BCR-ABL* transcripts at baseline and at the 3-month time-point allow calculation of either a *BCR-ABL* reduction rate (46) or a *BCR-ABL* halving-time (48). In their patient series, both indicators were reliable predictors of the benefit from various IM-based therapies. At the present time, we have not repeated *BCR-ABL/GUS*^{IS} measurements in our samples collected after 3 months of IM. Hence, we can't establish to what extent patients expressing high *BCR-ABL/GUS*^{IS} at diagnosis would also display modest reductions in their *BCR-ABL* ratio or in their *BCR-ABL* halving-times. Furthermore, while our results strongly suggest that patients expressing high levels of *BCR-ABL/GUS*^{IS} at diagnosis are unlikely to achieve optimal outcomes with standard dose IM, they have not established if these patients are generally unresponsive to TKIs or would benefit from higher (tolerability-adapted) doses of IM (12) or from 2G TKIs. Identification of baseline *BCR-ABL/GUS*^{IS} cut-offs for specific endpoints in wide

and uniformly treated patient cohorts will be necessary to address this issue that will be tackled by a prospective study beginning enrolment next year. Lastly, we used a previously reported statistical approach (49) to generate nomograms predicting the eight-year likelihood of achieving FFS when receiving standard dose IM according to *BCR-ABL/GUS*^{IS} expression at diagnosis and the Sokal or the Hasford scores (Supplementary Figure 5). As expected, these nomograms confirm that patients exhibiting elevated *BCR-ABL/GUS*^{IS} levels and included in the high Sokal or high Hasford categories display the highest risk of failing IM. These nomograms may be of clinical value for physicians in need of estimating the FFS probability of their patients.

In summary, our findings suggest that patients displaying higher levels of *BCR-ABL/GUS*^{IS} transcripts at diagnosis are less likely to benefit from standard dose IM. As achieving an OR according to the latest ELN recommendations is a mandatory goal for most newly diagnosed CML patients, baseline quantification of *BCR-ABL* expression may represent a useful parameter for physicians in need of discriminating patients likely to respond to IM from those that should receive alternative treatments. In the era of "personalized medicine", a molecular index reliably measurable at diagnosis might therefore prove useful in guiding the choice for the most appropriate first line treatment of CML (50).

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This manuscript is dedicated to Alessandra Aloisi and to Salvatore Berretta.

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Figure Legends

Figure 1. Comparison of *BCR-ABL/GUS*^{IS} levels at diagnosis in patients achieving an OR or failing IM. Patients were stratified according to their IM response as for the 2013 ELN criteria (optimal vs failure). *BCR-ABL*^{IS} transcripts - using *GUS* as a reference gene - were determined for each group and depicted as boxplots delimited by the 25th (lower) and 75th (upper) percentile. Horizontal lines above and below each boxplot indicate the 5th and 95th percentile, respectively. Thick lines in each boxplot represent median *BCR-ABL/GUS*^{IS} in each patient group. Patients failing IM displayed significantly higher *BCR-ABL* transcripts at diagnosis ($p<0.001$).

Figure 2. Eight-year estimates of OS (A), TFS (B), FFS (C) and EFS (D) in CML patients expressing high or low *BCR-ABL/GUS*^{IS} at diagnosis. The endpoint-specific *BCR-ABL/GUS*^{IS} thresholds identified in Table 1 were used to divide patients in two groups exhibiting high (dashed line) or low (solid line) *BCR-ABL* transcripts. Higher *BCR-ABL* levels were associated with significantly inferior rates of TFS ($p=0.029$), FFS ($p<0.001$) and EFS ($p<0.001$) but not OS. Vertical lines indicate censored patients.

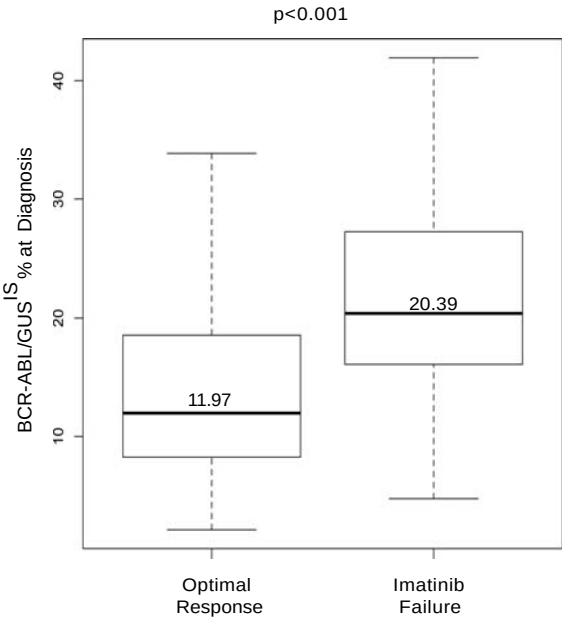

Figure 1

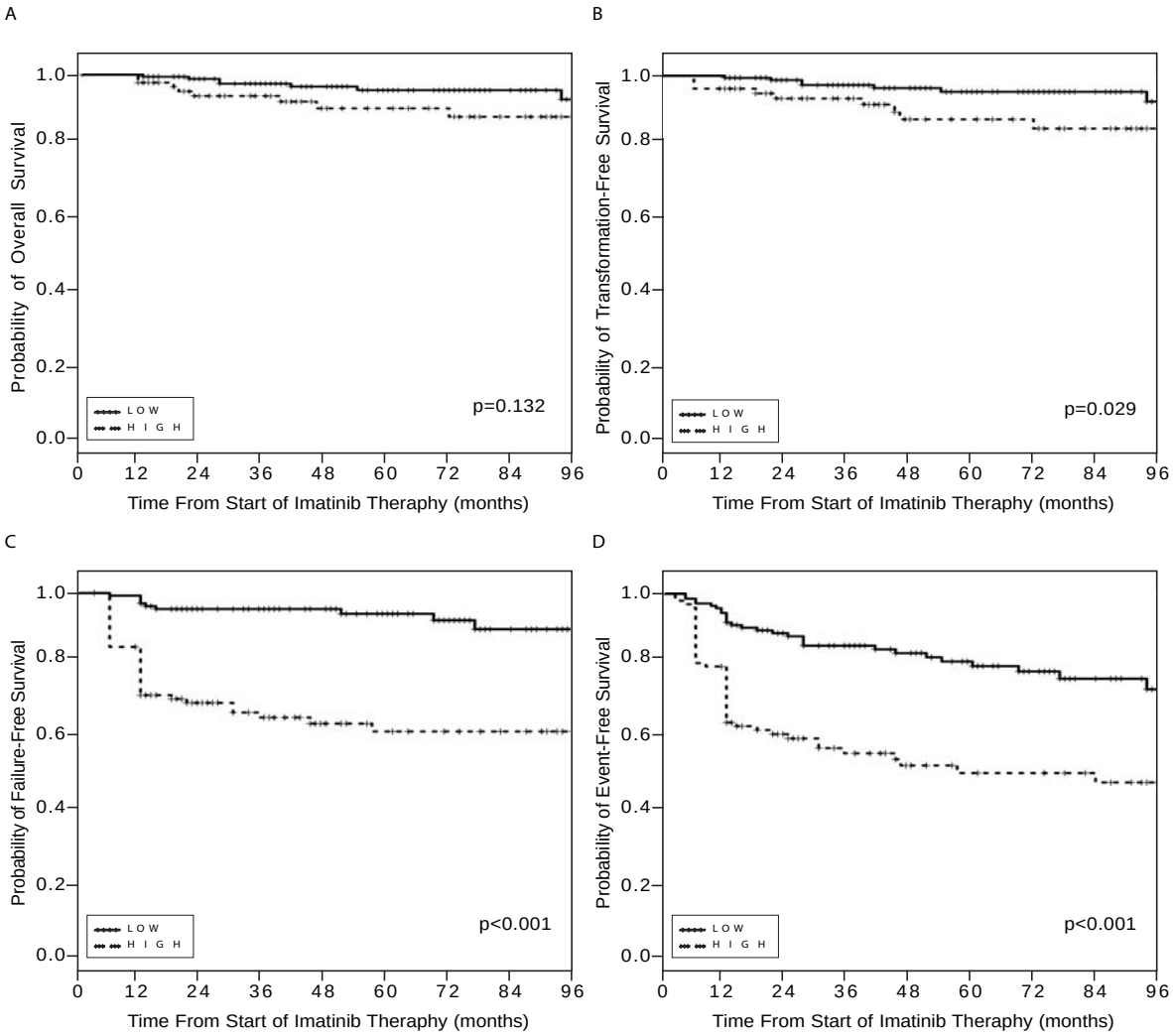

Figure 2

Table 1. ROC curves correlating *BCR-ABL/GUS*^{IS} levels at diagnosis with 8-year estimates of OS, TFS, FFS, EFS and Optimal Response

Outcome	Threshold (%)	Patients at Risk (%)	*Relative Risk*	*p*
OS	**18.55**			
Low Risk	≤18.55	162 (67.5)	1.14	0.132
High Risk	>18.55	78 (32.5)		
TFS	**18.79**			
Low Risk	≤18.79	165 (68.8)	2.03	0.029
High Risk	>18.79	75 (31.2)		
FFS	**14.89**			
Low Risk	≤14.89	135 (56.2)	3.82	<0.001
High Risk	>14.89	105 (43.8)		
EFS	**15.94**			
Low Risk	≤15.94	142 (59.2)	1.97	<0.001
High Risk	>15.94	98 (40.8)		
OR	**15.94**			
Low Risk	≤15.94	142 (59.2)	1.97	<0.001
High Risk	>15.94	98 (40.8)		

OS = Overall Survival; TFS = Transformation-Free Survival; FFS = Failure-Free Survival; EFS = Event-Free Survival; OR = Optimal Response

Table 2. **Internal validation experiments testing the performance of the FFS ROC values calculated with *GUS* or *ABL* as reference genes. Percentages indicate correct patient classification in the two subpopulations**

Reference gene	Randomization (times)	*Training Set* (n=192)	*Validation Set (n=48)*
GUS	50	100%	96%
ABL	50	94%	20%

FFS = Failure-Free Survival

Table 3. Association between different early molecular parameters and OS, TFS, FFS, EFS and Optimal Response

OVERALL SURVIVAL			
***BCR-ABL^{IS}* Transcript Threshold**	**Patients at Risk (%)**	***Relative Risk***	***p***
Ref. Gene GUS ***Diagnosis: 18.55%***	Patients (%)		
<18.55% (Low Risk)	162 (67.5)	1.14	0.132
>18.55% (High Risk)	78 (32.5)		
Ref. Gene ABL ***3 months: 10%***	Patients (%)		
<10% (Low Risk)	161 (78.5)	1.61	0.132
>10% (High Risk)	44 (21.5)		
Ref. Gene ABL ***6 months: 1%***	Patients (%)		
<1% (Low Risk)	149 (69.6)	2.7	0.025
>1% (High Risk)	65 (30.4)		
TRANSFORMATION-FREE SURVIVAL			
***BCR-ABL^{IS}* Transcript Threshold**	**Patients at Risk (%)**	***Relative Risk***	***p***
Ref. Gene GUS ***Diagnosis: 18.79%***	Patients (%)		
<18.79% (Low Risk)	165 (68.8)	2.03	0.029
>18.79% (High Risk)	75 (31.2)		
Ref. Gene ABL ***3 months: 10%***	Patients (%)		
<10% (Low Risk)	161 (78.5)	1.99	0.134
>10% (High Risk)	44 (21.5)		
Ref. Gene ABL ***6 months: 1%***	Patients (%)		
<1% (Low Risk)	149 (69.6)	2.2	0.021
>1% (High Risk)	65 (30.4)		

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FAILURE-FREE SURVIVAL			
***BCR-ABL^{IS}* Transcript Threshold**	**Patients at Risk (%)**	***Relative Risk***	***p***
Ref. Gene GUS			
Diagnosis: 14.89%	Patients (%)		
<14.89% (Low Risk)	135 (56.2)	3.82	<0.001
>14.89% (High Risk)	105 (43.8)		
Ref. Gene ABL			
3 months: 10%	Patients (%)		
<10% (Low Risk)	161 (78.5)	2.89	<0.001
>10% (High Risk)	44 (21.5)		
Ref. Gene ABL			
6 months: 1%	Patients (%)		
<1% (Low Risk)	149 (69.6)	7.48	<0.001
>1% (High Risk)	65 (30.4)		

EVENT-FREE SURVIVAL and OPTIMAL RESPONSE			
***BCR-ABL^{IS}* Transcript Threshold**	**Patients at Risk (%)**	***Relative Risk***	***p***
Ref. Gene GUS			
Diagnosis: 15.94%	Patients (%)		
<15.94% (Low Risk)	142 (59.2)	1.97	<0.001
>15.94% (High Risk)	98 (40.8)		
Ref. Gene ABL			
3 months: 10%	Patients (%)		
<10% (Low Risk)	160 (78.5)	1.99	<0.001
>10% (High Risk)	44 (21.6)		
Ref. Gene ABL			
6 months: 1%	Patients (%)		
<1% (Low Risk)	149 (69.6)	2.75	<0.001
>1% (High Risk)	65 (30.4)		

OS = Overall Survival; TFS = Transformation-Free Survival; FFS = Failure-Free Survival; EFS = Event-Free Survival; OR = Optimal Response

Table 4. Co-classification of different patient populations defined by distinct early molecular parameters

OVERALL SURVIVAL					
	BCR-ABL/GUS^{IS} diagnosis Threshold 18.55%		***Concordance***	***p***	***95% CI***
	<18.55% (Low risk)	>18.55% (High risk)			
BCR-ABL/ABL^{IS} 3 months	Patients (%)	Patients (%)			
<10% (Low risk)	110 (55%)	46 (23%)	67%	0.003	1.3 - 6.04
>10% (High risk)	20 (10%)	24 (12%)			
BCR-ABL/ABL^{IS} 6 months	Patients (%)	Patients (%)			
<1% (Low Risk)	112 (53.3%)	33 (15.7%)	71.9%	<0.001	2.6 - 10
>1% (High Risk)	26 (12.4%)	39 (18.6%)			
TRANSFORMATION-FREE SURVIVAL					
	BCR-ABL/GUS^{IS} diagnosis Threshold 18.79%		***Concordance***	***p***	***95% CI***
	<18.79% (Low risk)	>18.79% (High risk)			
BCR-ABL/ABL^{IS} 3 months	Patients (%)	Patients (%)			
<10% (Low risk)	112 (56%)	44 (22%)	67.5%	0.003	1.3 - 5.9
>10% (High risk)	21 (10.5%)	23 (11.5%)			
BCR-ABL/ABL^{IS} 6 months	Patients (%)	Patients (%)			
<1% (Low Risk)	114 (54%)	31 (14.8%)	72.3%	<0.001	2.6 - 10.2
>1% (High Risk)	27 (12.8%)	38 (18%)			

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FAILURE-FREE SURVIVAL

	BCR-ABL/GUS^{IS} diagnosis Threshold 14.89%		***Concordance***	***p***	***95% CI***
	<14.89% (Low risk)	>14.89% (High risk)			
BCR-ABL/ABL^{IS} 3 months	Patients (%)	Patients (%)			
<10% (Low risk)	93 (46.5%)	63 (31.5%)	61.5%	0.002	1.5 - 7
>10% (High risk)	14 (7%)	30 (15%)			
BCR-ABL/ABL^{IS} 6 months	Patients (%)	Patients (%)			
<1% (Low Risk)	99 (47.1%)	46 (21.9%)	70%	<0.001	3 -12.4
>1% (High Risk)	17 (8.1%)	48 (22.9%)			

OPTIMAL RESPONSE or EVENT-FREE SURVIVAL

	BCR-ABL/GUS^{IS} diagnosis Threshold 15.94%		***Concordance***	***p***	***95% CI***
	<15.94% (Low risk)	>15.94% (High risk)			
BCR-ABL/ABL^{IS} 3 months	Patients (%)	Patients (%)			
<10% (Low risk)	97 (48.5%)	59 (29.5%)	63%	0.001	1.5 - 6.9
>10% (High risk)	15 (7.5%)	29 (14.5%)			
BCR-ABL/ABL^{IS} 6 months	Patients (%)	Patients (%)			
<1% (Low Risk)	104 (49.5%)	41 (19.5%)	71.9%	<0.001	3.3 - 13.5
>1% (High Risk)	18 (8.6%)	47 (22.5%)			

CI = Confidence Intervals

AACR American Association for Cancer Research

Clinical Cancer Research

HIGH BCR-ABL/GUSIS LEVELS AT DIAGNOSIS OF CHRONIC PHASE CML ARE ASSOCIATED WITH UNFAVORABLE RESPONSES TO STANDARD-DOSE IMATINIB

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