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Short Communication Open Access

# Digital PCR (Dpcr) a Step Forward to Detection and Quantification of Minimal Residual Disease (MRD) in Ph+/BCR-ABL1 Chronic Myeloid Leukemia (CML)

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## Short Communication

Philadelphia-positive (Ph+), BCR-ABL1, chronic myeloid leukemia (CML) is a model of leukemia driven by a single, specific, chromosome translocation, the t (9;22) (q22;q11). This translocation, leading to a new, hybrid, leukemia-specific gene (*BCR-ABL1*) encoding for a deregulated tyrosine-kinase protein (p210), drives the leukemic transformation of hematopoietic stem cells [1-6] and induces the progression of the disease from the early chronic phase (CP) to the late blastic phase BP) which close the natural history of the disease.

In the 2000s, the introduction of Imatinib, the first tyrosine-kinase inhibitor (TKI) able to target the protein p210, significantly changed the fate of CML to fatal disease in real chronic disease [2]. Indeed, more than 80% of CML patients have a life expectancy close to that of the general population [7-11]. Imatinib and more recently the 2nd generation TKIs, namely Nilotinib, Dasatinib and Bosutinib, administered daily at the respective standard doses, by a progressive reduction of the Ph+ leukemic clone, can induce complete cytogenetic responses (CCyR) in more than 80% to 90% of cases, but, moreover, a major molecular response (MMR or $MR^{3.0}$=BCR-ABL1/ABL transcript level < 0.1% IS) and a deep molecular response (DMR) in 70% to 80% and 40% to 50% of patients, respectively [12].

The DMR ($MR^{4.0}$, $MR^{4.5}$ and $MR^{5.0}$), defined as BCR-ABL1 transcript level < 0.01% on the International Scale (IS) or as ABL transcript level (housekeeping gene) > 10,000 copies when BCR-ABL1 results undetectable (Table 1) [12-18], is now considered the most ambitious objective of therapy. Actually, we know that about 50% of patients achieving DMR are reported to maintain a stable treatment-free remission (TFR) after discontinuation of TKI treatment [19-22]. Thus, the current policy of CML treatment with TKIs is aimed to achieve at least a mayor molecular response (MMR or $MR^{3.0}$), as fast as possible, to prevent progression to blastic phase [3-6] and to gain the opportunity for treatment discontinuation when a DMR has been obtained and maintained for an as yet unspecified period of time [18-21,23-25]. Detecting and Monitoring the molecular response by quantitative polymerase chain reaction (qPCR) is therefore essential to measure minimal residual disease (MRD) [12-15] beyond the limits of CCyR and to optimize the management of CML patients on treatment with TKIs [3-5,12-18].

| | MMR | DMR | | |
|---|---|---|---|---|
| | $MR^{3.0}$ | $MR^{4.0}$ | $MR^{4.5}$ | $MR^{5.0}$ |
| Minimum sum of ABL1 transcripts irrespective of whether BCR-ABL1 is detected or not | - | 10.000 ABL1 copies | 32.000 ABL1 copies | 100.000 ABL1 copies |
| BCR-ABL1 IS levels for positive samples | ≤ 0.1% | ≤ 0.01% | ≤ 0.0032% | ≤ 0.001% |

**Table 1:** Current definition of MR classes following the last IS guide lines.

However, despite the international efforts to standardize the method, qPCR has some intrinsic limitations with regard to its limit of detection, the sensitivity to PCR-inhibitors, the reproducibility and moreover the loss of accuracy in the quantification of the low level of target. These features make it not optimal to select the best candidates for discontinuation of TKIs without relapse and to design personalized treatment programs, especially in the era of the more potent second generation TKIs that produce faster and deeper molecular responses. Therefore, overcoming the limits of qPCR should be seen as a necessary step forward in order to better manage the therapy with TKIs, to better select the candidates for TFR and to optimize the resources [23,25].

In recent years, digital PCR (dPCR) has emerged to provide a more sensitive and accurate detection of very low levels of disease that accounts for the increasing interest for its use in the clinic [26,27]. dPCR, based on the use of the most advanced instrumental platforms, is a new technology based on the partitioning of the sample in quantities on the scale of nanoliters or even picoliters by creating reaction chambers within specially designed chips or within sequestering reagents that become thousands of individual droplets. Therefore, the sample is randomly distributed into discrete partitions, in a way that some contain no nucleic acid template and others contain one or more template copies. A Poisson correction can be factored into the result to account for chambers that contain more than one molecule, and an absolute target sequence quantity can be estimated [27]. Moreover, dPCR provides an end-point measurement of absolute quantities of nucleic acids without the use of standard curves. Due to its characteristics, could the dPCR challenge the technical limits of qPCR and lead to a better sensitivity and accuracy of CML MRD detection and quantification?

Quant Studio 3D Digital PCR platform (Thermofisher) was applied to 350 samples of 120 CML patients, treated with TKIs and achieving MMR or DMR by qPCR, in order to obtain an absolute quantification of *BCR-ABL1*. Moreover, dPCR assay was applied also to 20 samples obtained by healthy subjects who served as healthy controls. dPCR

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results were expressed as BCR-ABL1 copies/µl of reaction and all samples was analyzed twice and in blind. Preliminary data show heterogeneous levels of BCR-ABL1 copies/µl assessed by dPCR among the CML patients grouping within the $MR^{3.0}$, $MR^{4.0}$, $MR^{4.5}$, and $MR^{5.0}$ class of molecular response. Importantly, the BCR-ABL1 transcript was quantifiable by dPCR also in those cases (n°=86) in whom it was below the level of detectability or undetectable by qPCR. Moving from $MR^{3.0}$ to $MR^{5.0}$ class, a progressive decrease of BCR-ABL1 copies/µl measured by dPCR was observed (Figure 1). In patients with $MR^{3.0}$, the median and range of BCR-ABL1 copies/µl assessed by dPCR [0.511 (0.232-1.692)] was significantly higher when compared with the median and range of BCR-ABL1 copies/µl of patients with $MR^{4.0}$ [0.313 (0.079-1.498) p=0.0003], $MR^{4.5}$ [0.135 (0.072-0.651) p<0.0001] or $MR^{5.0}$ [0.164 (0.074-0.539) p<0.0001] (Figure 1). In the case of healthy controls and blanks, the values of BCR-ABL1 copies/µl were comprised between 0.000-0.061 and 0.000-0.060, respectively, and they were statistically different from the ones measured in the $MR^{3.0}$, $MR^{4.0}$, $MR^{4.5}$ and $MR^{5.0}$ patients' group (p<0,0001; p<0,0001; p=0.02 and p<0,0001 respectively). No statistically significant differences were observed comparing $MR^{4.0}$ vs $MR^{4.5}$ or $MR^{5.0}$, or $MR^{4.5}$ vs $MR^{5.0}$. We did not find any linear correlation (R=0.196) between the BCR-ABL1 copies/µl assessed by dPCR and the values of BCR-ABL1/ABL1% IS (Figure 2a). Similarly, we did not find any linear correlation (R=0.184) when comparing the BCR-ABL1 copies/µl as assessed by dPCR with the absolute copy number of BCR-ABL1 transcripts as assessed by qPCR, when detectable (Figure 2b). However, examining the distribution of the cases in the latter analysis, two distinct populations emerged. In order to identify the sub-population showing a different distribution, a case by case analysis was performed and it revealed a linear correlation (R=0.68) for the cases with an absolute copy number of BCR-ABL1 transcript >80 as assessed by qPCR (Figure. 2c). This population included 11 samples, all belonging to the $MR^{3.0}$ class. Using the one-way ANOVA test, including all the patients with detectable and undetectable BCR-ABL1 transcript by qPCR, we find a correlation between dPCR BCR-ABL1 levels and the $MR^{3.0}$ class, while no correlation has been found when dPCR BCR-ABL1 levels were compared with the $MR^{4.0}$, $MR^{4.5}$ and $MR^{5.0}$ class, separately analyzed. The ROC analysis indicated the value of 0.468 BCR-ABL1 copies/µl as the value below which the patients with lower levels of minimal residual disease might be dissected (specificity=71%, sensitivity=78%; AUC=0,79). Below the value of 0.468 BCR-ABL1 copies/µl fell 246/350 (70%) samples, of whom: 48/52 (92%) with $MR^{5.0}$; 88/94 (94%) with $MR^{4.5}$; 72/98 (73%) with $MR^{4.0}$; and 38/106 (36%) with $MR^{3.0}$. These data suggest that high amounts of BCR-ABL1 transcript are well quantified by qPCR, while minimal ones may be better detected and quantified using a dPCR approach. This hypothesis is confirmed by different statistical analysis. According to our data, dPCR analysis seems to be useful for a better stratification of patients in DMR. This is very important because DMRs are patients potentially eligible for TKI discontinuation and they could be better identified if tested for MR both by qPCR and by dPCR.

More recently, Droplet Digital™ PCR system (Bio-Rad) was used to test cDNA samples from CML patients, in chronic phase, treated with TKIs, within the ENEST1st [28] or EURO-SKI [29] clinical trials and out of controlled clinical trials [30]. All these studies unanimously conclude in saying that the dPCR is more sensitive and accurate than the qPCR for the quantification of BCR-ABL1 transcript, and that it can contribute to a better identification and stratification of patients with deep molecular response [28-30]. The data obtained in this preliminary cohort of patients are limited and a systematic comparative study should be done in the future to understand the power of dPCR in discriminating the clinical relevance of different levels of minimal

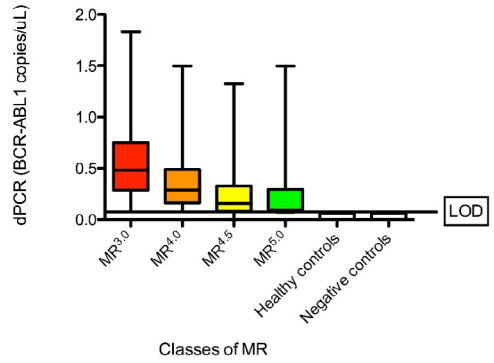


**Figure 1:** Distribution of BCR-ABL1 copies/µl measured by dPCR in the patients belonging to the $MR^{3.0}$, $MR^{4.0}$, $MR^{4.5}$, $MR^{5.0}$ classes of response as determined by qPCR.

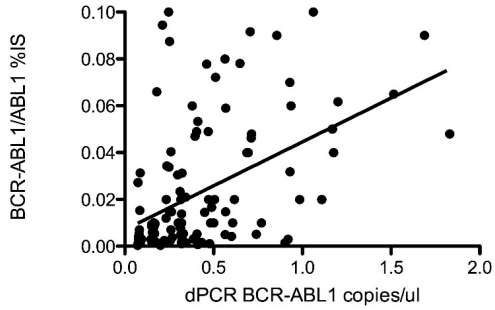


**Figure 2(a):** Linear regression analysis between qPCR BCR-ABL1/ABL1 IS %, and dPCR BCR-ABL1 copies/µl. MR undetectable were excluded from the linear regression analysis.

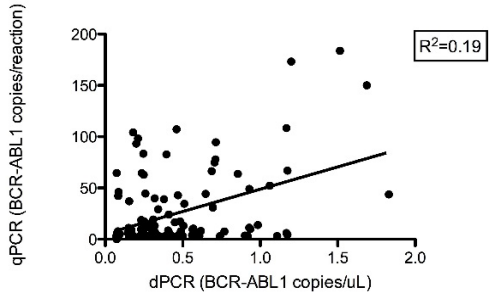


**Figure 2(b):** Linear regression analysis between qPCR BCR-ABL1 copies/reaction and dPCR BCR-ABL1 copies/µl. MR undetectable were excluded from the linear regression analysis.

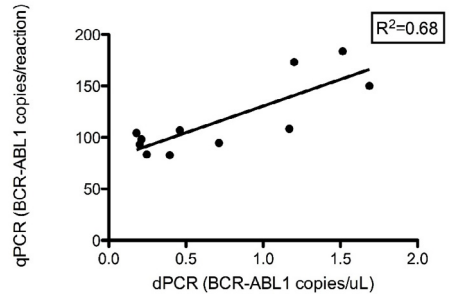


**Figure 2(c):** Linear regression analysis between qPCR BCR-ABL1 copies/reaction >80 copies and dPCR BCR-ABL1 copies/µl.

residual disease and in detecting the best responders. This is an important point and it is very likely that the criteria for scoring MR as we know them today will be revised in the light of the systematic use of dPCR.

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