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RESEARCH

Digital PCR (dPCR) is able to anticipate the achievement of stable deep molecular response in adult chronic myeloid leukemia patients: results of the DEMONSTRATE study

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Abstract

Chronic Myeloid Leukemia (CML) is marked by the BCR::ABL1 fusion gene. Monitoring tyrosine kinase inhibitor (TKI) therapy response is crucial for treatment management, thus, limitations in Reverse Transcription quantitative PCR's (RT-qPCR) accuracy and sensitivity led to the exploration of alternative methods like digital PCR (dPCR). This study evaluated dPCR efficacy in detecting Minimal Residual Disease (MRD) in CML patients undergoing TKI therapy. 79 CML patients were enrolled (NP 3809 clinical trial), with samples analysed using both methods. The achievement and stability of Deep Molecular Response (DMR) were assessed over a 2-year period following the first DMR achievement. A comparative statistical analysis of MRD and DMR attainment, stability, and potential TFR achievement using both RT-qPCR and dPCR was conducted, supported by chi-squared tests, Fisher's exact tests, and Kaplan–Meier analysis. In 69/79 patients, dPCR either anticipated or coincided DMR achievement as compared to RT-qPCR. Among them, 52/69 achieved a stable DMR according to RT-qPCR, while 44/69 according to dPCR. Thus, dPCR capability to anticipate or coincide the achievement of a stable DMR resulted with $p = 0.0012$ and $p = 0.0017$, respectively. Transcript type and TKI choice did not influence DMR achievement or stability by either method. These findings highlight dPCR as a sensitive and accurate tool for monitoring MRD in CML patients, providing information for treatment management decisions, and potentially enhancing the selection of candidates for treatment-free remission. Further standardization of dPCR methodologies is warranted to leverage their benefits in clinical practice.

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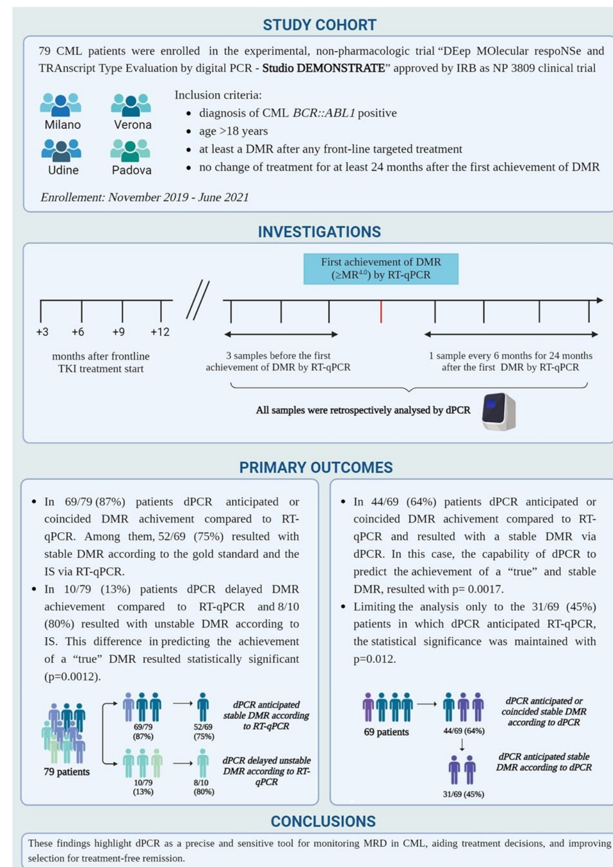
Graphical abstract



Keywords CML · dPCR · MRD · TKIs · Transcript type

Introduction

Chronic Myeloid Leukemia (CML) is a myeloproliferative disease driven by a reciprocal translocation between chromosome 9 and 22 (the so-called Philadelphia chromosome), resulting in the formation of a leukemogenic hybrid gene (*BCR::ABL1*). Sites of breakpoint in the *ABL1* gene (chromosome 9) and in the *BCR* gene (chromosome 22) can occur in different regions, giving arise to oncogenic proteins with different molecular weight [1]. The majority of CML patients harbour one of the two splicing variants of the M-bcr (p210) fusion protein, i.e. e13a2 or e14a2 (b2a2 or b3a2).

Several studies reported differences in biological characteristics (e.g. lower platelet count and higher WBC count in e13a2 patients) [2] and response to Tyrosine Kinase Inhibitor (TKI) when considering the transcript types [3]. It was also found that e13a2 transcript type was associated with a slower rate of molecular response [4, 5], a reduced probability of stable deep response [6–8], when assessed by conventional RT-qPCR, and a lower probability of treatment-free remission (TFR) success [8–10], but not with a different survival [11].

A recent analysis in a series of > 45,000 newly diagnosed CML from different countries showed that the proportion of transcript types does not differ by chance, being e13a2 more frequent in males and decreasing with age [12]. However, it is debated if these differences are more "biological" (i.e. due to a different immunogenicity of transcript types [13]), or "technical" (i.e. due to the different amplification of transcripts by conventional RT-qPCR [14, 15]), considering that the response to TKIs therapy is monitored by RT-qPCR, normalised with a reference gene.

Deep Molecular Response (DMR) is defined as a measurable MR if ≤ 0.01% *BCR::ABL1*%IS, or undetectable *BCR::ABL1* with at least 10,000 copies of the reference *ABL1* gene, according to the most recent recommendations of the International Expert Panel [16, 17]. DMR is scored as MR4.0 if ≤ 0.01% *BCR::ABL1*%IS, MR4.5 if ≤ 0.0032%

BCR::ABL1%IS, or MR5.0 if ≤ 0.001% *BCR::ABL1*%IS. Minimum sum of *ABL1* reference gene transcripts, irrespective of whether *BCR::ABL1* will be detected or not, are 10,000, 32,000 and 100,000 for MR4.0, MR4.5 and MR5.0, respectively. In Italy, MR assessment takes place in a standardized manner through the accredited and certified Laboratories involved in the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) LabNet Network.

Despite the efforts to standardize the method, RT-qPCR has intrinsic limitations regarding its accuracy and sensitivity. These limits are relevant in patients with very low level of disease, since an accurate detection of minimal residual disease (MRD) is of a paramount importance to select the best candidates for TFR. Therefore, overcoming these limits should be seen as a necessary step forward to better manage TKIs therapy. Digital PCR (dPCR) challenges the technical limits of RT-qPCR [18] demonstrating in several studies its capability to accurately quantify *BCR::ABL1* transcript [19–24]. Preliminary results of our studies showed that a value of < 0.468 *BCR::ABL1* copies/μL, indicated by ROC analysis, may identify patients with lower MRD levels who also have a better probability of maintaining TFR status [25].

It has been hypothesized that a different amplification of PCR products may partly account for the differences in molecular response between transcript types, especially for deep responses. The dPCR is expected to not suffer from such a limitation by providing an absolute measure of *BCR::ABL1* copies.

To define the potential advantages of dPCR in detecting and monitoring of CML-MRD not only in patients already in DMR, dPCR was used to assess the absolute levels of *BCR::ABL1* transcript in CML patients treated with TKIs from diagnosis to the first DMR achievement. Although no standardized definition of "stable DMR" exists, there is evidence to suggest that ≥ 2 years of sustained molecular response ≥ MR4.0 is associated with superior TFR success [26, 27] and expert panels suggested that treatment discontinuation is feasible in patients that achieved and maintained a molecular response ≥ MR4.0 for ≥ 2 years [28]. Therefore, the evaluation of kinetics of molecular responses over a 2 year period after the first MR4.0 achievement was chosen for the design of this study.

The results of dPCR and RT-qPCR were compared and related with clinical and biological characteristics.

Materials and methods

Patients, sampling and MRD monitoring by RT-qPCR

A total of 79 CML patients were enrolled and analysed in the experimental, non-pharmacologic trial "DEep MOlecular respoNSe and TRAnscript Type Evaluation by digital PCR—Studio DEMONSTRATE" approved by IRB of the enrolling Centres of Verona, Padova, Milano, and Udine (all in Italy) in July 2019 as NP 3809 clinical trial. The inclusion criteria were: diagnosis of CML *BCR::ABL1* positive, age > 18 years, having obtained after front-line targeted treatment (any TKIs, any dose) at least a DMR following the IS, having maintained the same TKIs at the same dose for at least 24 months after the first DMR achievement. The first patient was enrolled in November 2019, while the last one in June 2021. The studies were conducted in accordance with the Declaration of Helsinki and the enrolled patients gave their written informed consent. Data concerning sex, age, *BCR::ABL1* transcript type, TKIs administration (Type of TKIs, dose, and therapy duration), time of DMR achievement, stability of DMR following RT-qPCR, and TKIs discontinuation (TFR achievement) were recorded. The timeline of the investigated samples is reported in Fig. 1. Briefly, per patient we collected: four samples during the first year after diagnosis, three samples (one every three months) before the achievement of the DMR according to RT-qPCR, the sample relative to the DMR achievement, and one sample every six months for the following 2 years after the obtaining of the DMR. The same samples were analysed simultaneously by dPCR.

RNA extraction and retrotranscription

The RNA samples were retrospectively collected. Only RNA samples adequately managed and stored at −80 °C were considered. At the time of sampling, RNA extraction was performed after PB centrifugation on PB cells at the enrolling Centres by NucleoSpin RNA plus (Macherey Nagel, Düren, Germany), following the manufactures' instructions. This procedure was approved for clinical diagnostic application in Italy. Extracted RNA was immediately quantified by Qubit RNA High sensitivity kit (Thermo Fisher Scientific, Waltham, Massachusetts) and used for the RT-qPCR, as described below.

An aliquot of RNA was centralized in Brescia Centre via express courier in dry ice and subsequently retrotranscribed by the following method.

1 μg of total RNA in 10 μl of DNA- RNA-free H2O, in line with Labnet guidelines, was retrotranscribed starting with an incubation at 70 °C for 10', followed by ice incubation. Then, 100U of Reverse transcriptase (Superscript I or II), 1 mM of dNTP, 10 mM of DTT, 25 μM of Random hexamers, 20U of RNAse inhibitor and RT buffer (according to the enzyme used) were added to a final volume of 20 μl. This step was followed by three subsequent incubations: at room temperature for 10', 42 °C for 45', 99 °C for 3' with a final holding at 4 °C. The cDNA was quantified using

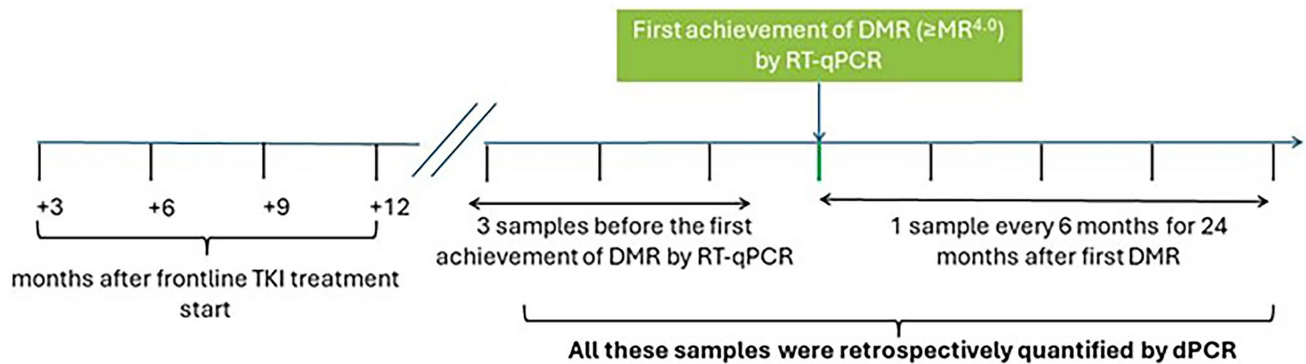


Fig. 1 Time schedule of retrospectively and prospectively analysed samples. In the study, all the samples collected in the first year after CML diagnosis and during the TKI treatment were considered. Retrospectively, 3 samples before the DMR achievement following the RT-qPCR quantification, together with 1 sample every 6 months for 2 years after the first DMR were recovered and analysed

Qubit ssDNA kit (Thermo Fisher Scientific, Waltham, Massachusetts) and then stored at −20 °C until analysis. The retrotranscription was performed as described in Bernardi S. et al., 2019 [25] in order to guarantee the normalization of the amount of RNA considered during the analysis.

RT-qPCR

BCR::ABL1 transcript quantification was performed using the automated Xpert Ultra BCR-ABL1 Monitor™ Cepheid (Milan, Italy) method and calibrated for the transcript of *ABL1* reference gene and *BCR::ABL1* target gene, with PCR sensitivity of 5.0 (> 250,000 *ABL1* copy numbers). Samples were analysed according to the manufacturer's instruction and following the internationally standardized methods of MRD monitoring in CML. Indeed, molecular responses were expressed and reported as *BCR::ABL1%* on a log scale, where 0.1%, 0.01%, 0.0032%, and 0.001%, corresponding to MR3.0, MR4.0, MR4.5, and MR5.0, respectively. The minimum sum of *ABL1* reference gene transcripts, irrespective of whether *BCR::ABL1* was detected or not, should be 10.000 for MR4.0, 32.000 for MR4.5, and 100.000 for MR5.0.

dPCR

BCR::ABL1 quantification via dPCR was performed on a QuantStudio™ Absolute Q Digital PCR System by Life Technologies. Briefly, for each sample, a 9 µl final volume of reaction mix was prepared, containing 1.8 µl of Absolute Q™ DNA Digital PCR Master Mix (5X) (Thermo Fisher Scientific, Waltham, Massachusetts), 0.45 µl of 20X TaqMan-MGB-FAM probe assay, previously published [25, 29], 5 µl of cDNA and 1,75 µl of nuclease-free water (Qiagen). The reaction mix was loaded in the absolute Q MAP16 plate and 15 µl of Absolute Q Isolation Buffer (Thermo Fisher Scientific, Waltham, Massachusetts) were added. The thermocycling conditions were the following: 95 °C for 8', 43 cycles at 90 °C for 15'', 60° for 1', followed by a final extension step at 60 °C for 2'. Raw data of fluorescence emission and target quantification were analysed by Applied Biosystems™ QuantStudio™ Absolute Q digital PCR Software (Version 6.3.0).

Statistical analysis

A descriptive statistical analysis was carried out to compare MRD and DMR attainment using RT-qPCR and dPCR, as well as DMR stability and potential TFR achievement. For dPCR, stability was indicated by a *BCR::ABL1* transcript value below 0.468 copies/µL according to our previous analysis [25]. For this purpose, the median and range were used to describe time variables, while analysis of categorical variables was conducted using Chi-squared or Fisher test according to data characteristics. Finally, the DMR duration according to RT-qPCR and dPCR in the *pre*, *equal* and *post* cases have been compared via Kaplan–Meier plots, with associated log-rank test to assess the statistical significance. All statistical analysis were performed using GraphPad Prism software (version 8.4.3) and RStudio software (version 2023.12.0).

Results

The main characteristics of the enrolled patients are reported in Table 1.

Patients were grouped in 3 categories according to timing of the DMR. Specifically, we investigated (i)

Table 1 Features of CML patients evaluated into the study

N° CML patients = 79	
Sex (Male–Female) (*n* (%))	38—41 (48%—52%)
Age at diagnosis (Median (range))	52y (18—85)
Transcript type (*n* (%))	
e13a2	16 (20%)
e14a2	53 (67%)
e13a2/e14a2	7 (9%)
NA	3 (4%)
Therapy (*n* (%))	
Imatinib	48 (61%)
Dasatinib	18 (23%)
Nilotinib	13 (16%)
First MR class at DMR achievement	
MR4 (I, D, N)	59 (75%) (35, 14, 10)
MR4.5 (I, D, N)	16 (20%) (11, 3, 2)
MR5 (I, D, N)	4 (5%) (2, 1, 1)
Time to first DMR (Mean ± SD)	
Total	18 m ± 13
Imatinib	20 m ± 12
Dasatinib	13 m ± 10
Nilotinib	17 m ± 17

DMR Deep molecular response; *NA* Not available; *MR* Molecular response; *n* number; *y* years; *m* months; *I* Imatinib; *D* Dasatinib; *N* Nilotinib; *SD* Standard deviation

whether the dPCR anticipated (dPCR *pre* RT-qPCR category), coincided (dPCR *equal* RT-qPCR category), or delayed (dPCR *post* RT-qPCR category) DMR achievement compared to RT-qPCR, (ii) for these categories, we evaluated if DMR was maintained or not for two years according to dPCR only, RT-qPCR only, both, or neither (Fig. 2). For RT-qPCR, the DMR was considered stable if the transcript value was below 0.01% for the IS, while, for dPCR, stability was indicated by a *BCR::ABL1* transcript value below 0.468 copies/µL for any time point after the DMR achievement and for at least 24 months. DMR was considered unstable if, at any point during a two-year period, the values exceeded these limits, even if it occurred just once.

Following the grouping strategy, 44/79 (56%) patients were in dPCR *pre* RT-qPCR, 25/79 (31%) patients were in dPCR *equal* RT-qPCR, and 10/79 (13%) were in dPCR *post* RT-qPCR. Particularly, dPCR anticipated or coincided the DMR achievement in 24 and 11 patients presenting MRD stability by both the techniques, respectively. Among those with MRD stability detected only by dPCR, the same technique anticipated DMR achievement in 7 patients and coincided in 2 patients. While dPCR anticipated the DMR achievement in 9 patients and coincided in 8 patients among those with MRD stability detected only by RT-qPCR. Finally, dPCR anticipated or coincided DMR achievement in only 4 patients each presenting MRD instability by both techniques, meaning that most patients in these categories present stable DMR by at least one technique. Moreover, only 1 patient presenting MRD stability by both the techniques was postponed by dPCR. Eight patients were postponed by dPCR and presented instability by both the techniques, meaning that most patients in this category present unstable DMR.

First, we assessed the presence of a correlation between the timing of the DMR achievement according to dPCR versus RT-qPCR and the stability of DMR, initially assessed with RT-qPCR (independently from dPCR results) and then with dPCR (independently from RT-qPCR results). To evaluate if dPCR can early identify patients who are not deep responders, we performed the statistical analysis considering the dPCR *post* RT-qPCR category alone. In our cohort 69/79 (87%) patients were in dPCR *equal* or *pre* RT-qPCR. Among them, 52/69 (75%) resulted with stable DMR according to the gold standard and the IS via RT-qPCR. Conversely, among 10/79 (13%) patients in dPCR *post* RT-qPCR, 8/10 (80%) resulted with unstable DMR by RT-qPCR and IS. This difference in predicting the achievement of a "true" DMR resulted statistically significant ($p = 0.0012$, Fig. 3A), in favour of dPCR. Kaplan–Meier curve confirmed these results ($p = 0.00071$,

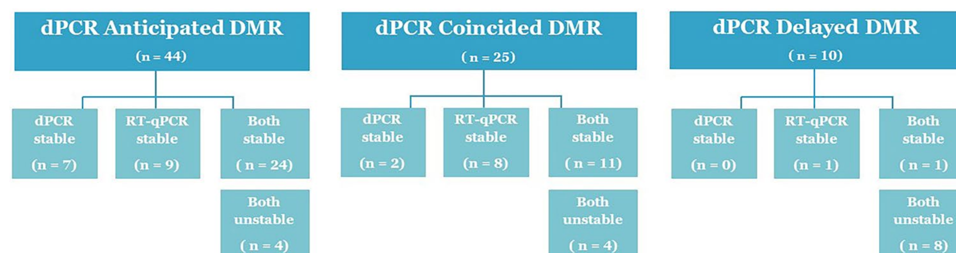


Fig. 2 Patients were grouped according to the timing of the DMR achievement by dPCR versus RT-qPCR (anticipated, coincided, delayed). Then, patients were sub-grouped based on DMR stability via dPCR only, RT-qPCR only, both, or neither. (dPCR = digital PCR; DMR = deep molecular response; RT-qPCR = real time quantitative PCR, n = number)

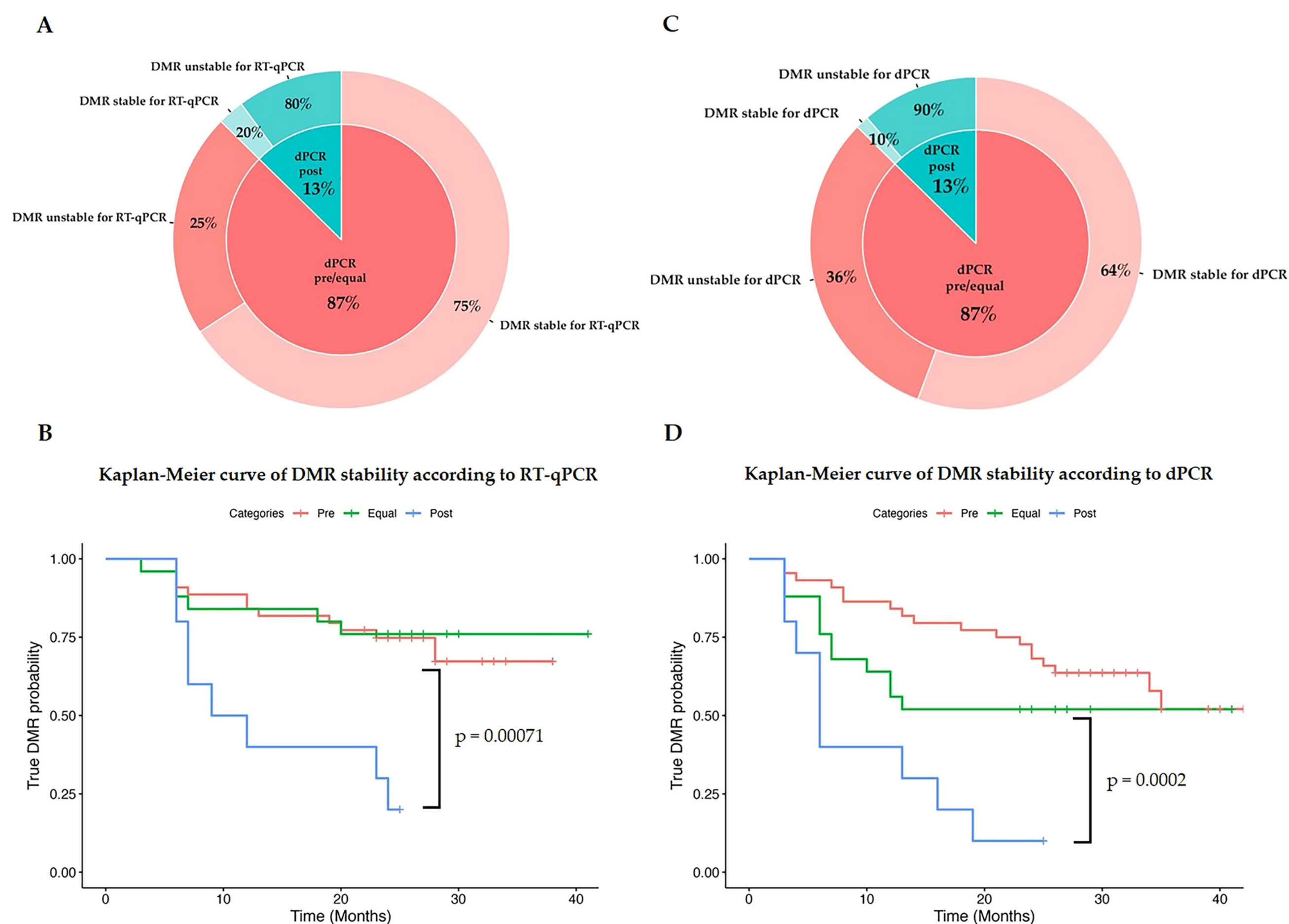


Fig. 3 **A** Graphical distribution of patients presenting a DMR anticipated/coincided/delayed by dPCR and related DMR stability assessed by RT-qPCR. **B** Kaplan–Meier curve reporting the probability of DMR stability according with RT-qPCR detection in case of DMR achievement anticipated, coinciding, or postponed by dPCR. **C** Graphical distribution of patients presenting a DMR anticipated/coincided/postponed by dPCR and related DMR stability assessed by dPCR. **D** Kaplan–Meier curve reporting the probability of DMR stability according with dPCR detection in case of DMR achievement anticipated, coinciding, or postponed by dPCR. (DMR = Deep Molecular Response; dPCR = digital PCR; pre = dPCR pre RT-qPCR; Equal = dPCR equal RT-qPCR; post dPCR post RT-qPCR)

Fig. 3B). On the other hand, 44/69 (64%) of patients in dPCR *pre* RT-qPCR or dPCR *equal* RT-qPCR, resulted with a stable DMR via dPCR, conventionally estimated as accurate and sensitive. In this case, the capability of dPCR to predict the achievement of a "true" and stable DMR, resulted with $p = 0.0017$. Additionally, limiting the analysis only to the 31/69 (45%) patients in dPCR *pre* RT-qPCR, the statistical significance was maintained with $p = 0.012$, confirming the capability of dPCR the anticipate a "real" DMR. To note that in this group were excluded 3 patients presented unstable DMR by RT-qPCR because of just one time point resulted with MR3.0 at 0.011 IS% (Fig. 3C), so at the limit of the DMR. Kaplan–Meier curve confirmed these results ($p = 0.0002$, Fig. 3D).

Analysing the depth of the first DMR, 59/79 patients (75%) achieved the DMR in MR 4.0, 16 (20%) in MR 4.5, and 4 (5%) in MR 5.0. dPCR anticipated the achievement of DMR in 19/20 (95%) patients presenting a "very deep" DMR, meaning MR 4.5 or MR 5.0 at the first time point.

In terms of time, in the group of patients in dPCR *pre* RT-qPCR, the median time to achieve first DMR was 10 months (range 3–39) by dPCR compared to 18 months (range 7–50 months) by RT-qPCR. Conversely, in dPCR *post* RT-qPCR, the median time to achieve first DMR was 36 months (range 13–68) by dPCR compared to 25 months (range 6–62 months) by RT-qPCR. In the 25 patients in dPCR *equal* RT-qPCR, the median time to achieve first DMR was 7 months (range 5–52).

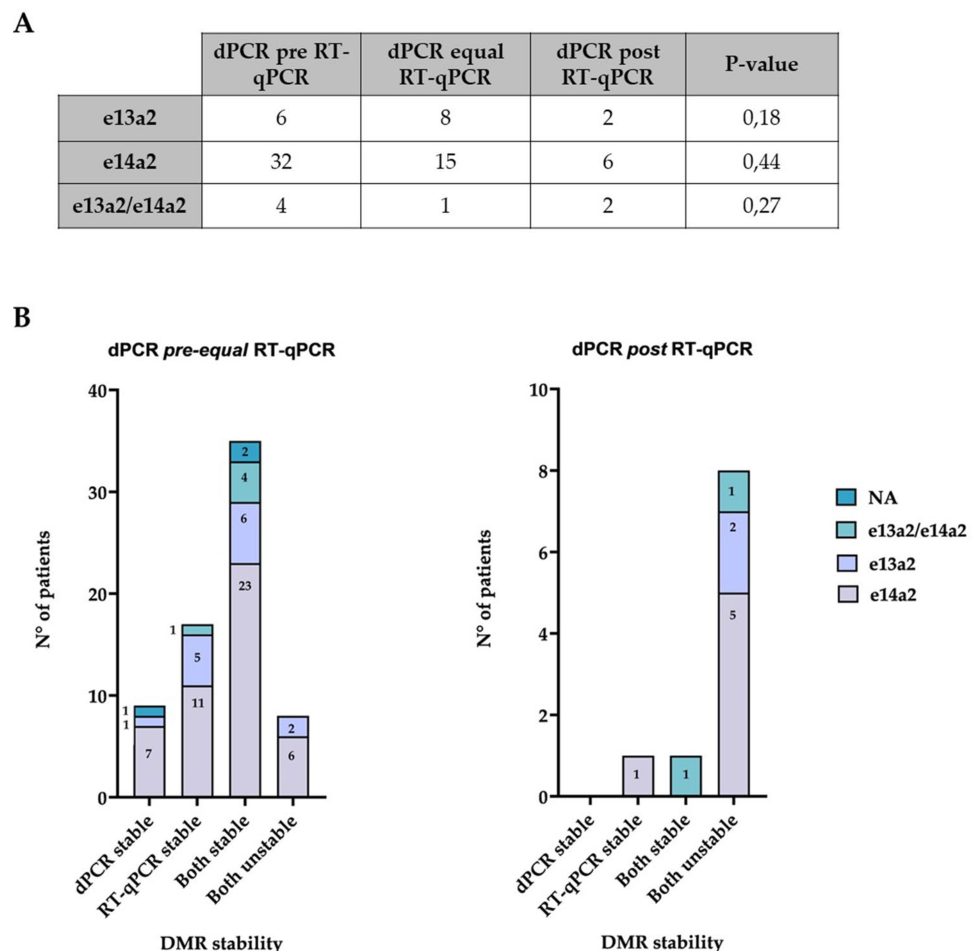
One of the primary endpoints of the study was to evaluate whether the transcript type could be correlated with the response to TKIs, the rate of molecular response or reduced probability of stable deep response. Therefore, we analysed the distribution of the transcript type within the above

mentioned categories. In our cohort, 16/79 (20%) had the e13a2, 53/79 (67%) the e14a2, 7/79 (9%) presented both transcripts, and in 3 patients transcript type was unknown.

To find a correlation between the transcript type and the dynamic of achievement of the DMR, we evaluated the segregation of the transcript types within the categories of dPCR *pre* RT-qPCR, dPCR *equal* RT-qPCR, and dPCR *post* RT-qPCR. Statistical analysis confirms that the distribution of the transcript types within the categories display no correlation, as reported in Fig. 4A.

We then considered the transcript distribution within three categories together with the DMR stability according dPCR and RT-qPCR (Fig. 4B). In dPCR *pre* and *equal* RT-qPCR categories, 6, 23, 4, and 2 patients with DMR stability according to both techniques presented e13a2, e14a2, both and unknown transcript type, respectively. The only patient in dPCR *post* RT-qPCR presenting MRD stability by both the techniques had both transcripts. dPCR anticipated or coincided the DMR achievement in 1, 7, and 1 patient having a stable DMR only by dPCR and presenting e13a2, e14a2, and unknown transcript type, respectively. dPCR anticipated or coincided the DMR achievement in 5, 11, and 1 patient having a stable DMR only by RT-qPCR and presenting e13a2, e14a2, and both the transcript type, respectively. The single patient in dPCR *post* RT-qPCR and presenting DMR stability only by RT-qPCR had e14a2 transcript. Finally, among patients presenting instability by both PCR technologies, dPCR anticipated or coincided the DMR achievement in 2 and 6 patients presenting e13a2 and e14a2, respectively, while dPCR delayed the DMR achievement in 2, 5, and 1 patient presenting e13a2, e14a2, and both the transcript types, respectively.

The TKIs administered to the patients were considered to assess their influence on dPCR and RT-qPCR capability to detect transcript slope. In our cohort, 48/79 (61%) were treated with imatinib, 13/79 (16%) with dasatinib, and 18/79 (23%) with nilotinib. dPCR delayed the achievement of DMR in 5/48 (11%) and 5/31 (16%) patients under imatinib and 2nd generation TKIs, respectively. Whereas, in 43/48 (89%) patients under imatinib and 26/31 (84%) patients under 2nd generation TKIs, dPCR anticipated or coincided the achievement of DMR. Statistical analysis showed no correlation between the category of DMR achievement and the type of TKI (Fig. 5A). This indicates


A

	dPCR pre RT-qPCR	dPCR equal RT-qPCR	dPCR post RT-qPCR	P-value
e13a2	6	8	2	0,18
e14a2	32	15	6	0,44
e13a2/e14a2	4	1	2	0,27

Fig. 4 **A** Distribution of the different transcript types (e13a2, e14a2, and both) within the categories. **B** Distribution of the transcript types within the dPCR pre and equal RT-qPCR and dPCR post RT-qPCR categories. Each category is divided following to DMR stability according to dPCR, RT-qPCR, both or neither. (dPCR = digital PCR; RT-qPCR = real time quantitative PCR; DMR = deep molecular response; NA = not available)

A

	dPCR *pre* and *equal* RT-qPCR	dPCR *post* RT-qPCR	P-value
Imatinib	43	5	0,45
2nd generation TKIs	26	5	

B

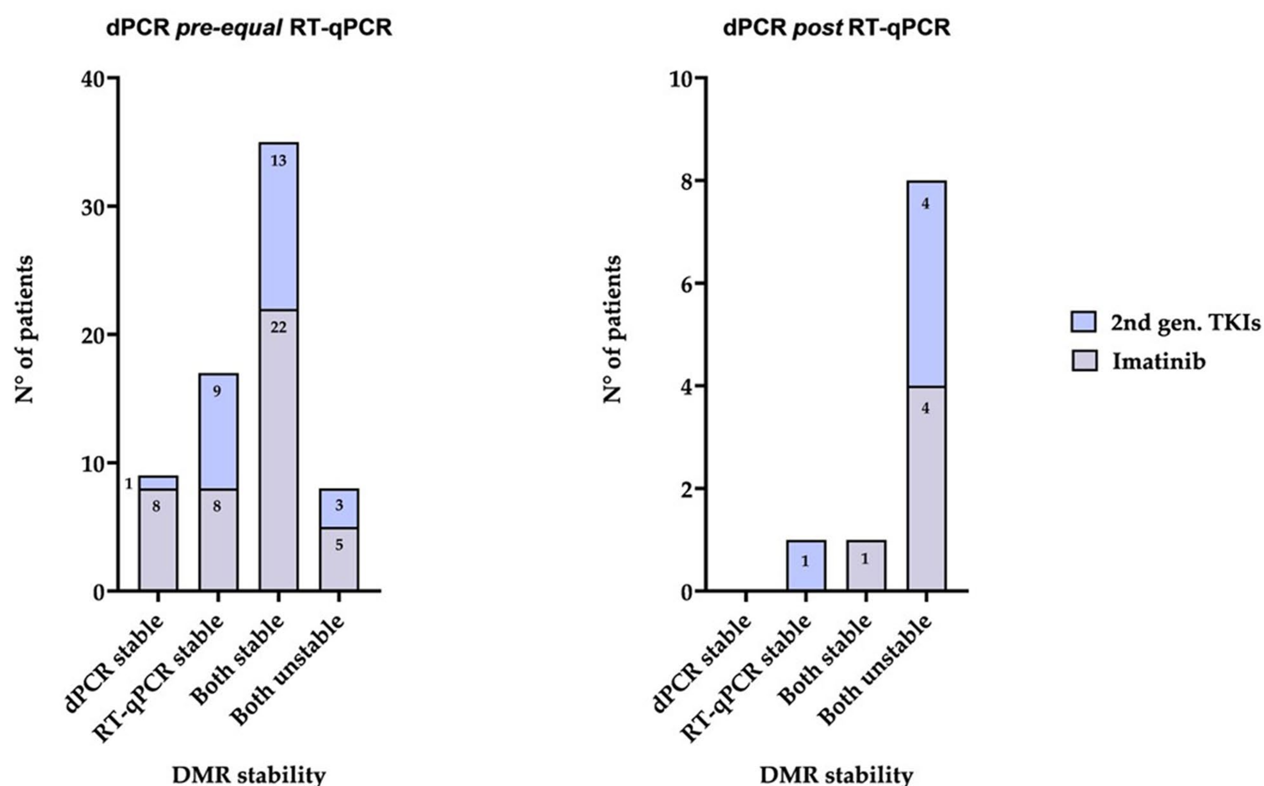


Fig. 5 **A** Distribution of patients according to TKIs within the categories. **B** Distribution of the patients according to TKI administered within the dPCR pre and equal RT-qPCR and dPCR post RT-qPCR categories. Each category is divided following to DMR stability according to dPCR, RT-qPCR, both or neither. (dPCR = digital PCR; RT-qPCR = real time quantitative PCR; DMR = deep molecular response; TKIs = tyrosine kinase inhibitors)

that the effectiveness of dPCR and RT-qPCR in anticipating, coinciding, or delaying DMR was not influenced by the type of TKI administered.

In terms of stability, the category of dPCR *post* RT-qPCR was not included in the statistical analysis due to the small number of patients in this group. Considering the category of dPCR *pre* and *equal* RT-qPCR, patients were evenly distributed into groups based on DMR stability as determined by dPCR and/or RT-qPCR (Fig. 5B). Again, statistical analysis showed no correlation between the stability of the DMR and the type of TKI administered.

Discussion

The use of dPCR in CML has been explored within many clinical trials focused on evaluating the capability of this technique to better assess DMR in patients eligible for TFR [22, 24, 26]. The majority of these trials were retrospective and only few ones perspective. To our knowledge, this is the first attempt to evaluate the benefits of dPCR in terms of timely DMR achievement and its stability. Variables affecting TFR achievement, such as transcript type, TKI

and DMR depth, were also considered in this analysis. Most of enrolled patients (44/79, 56%) resulted in the dPCR *pre* RT-qPCR group. This is an interesting finding since dPCR is considered as a very sensitive technique, able to detect a higher amount of marker in case of rare target [1, 2]. Nevertheless, dPCR accuracy and capability to precisely quantify *BCR::ABL1* transcript in CML samples resulted strongly impacting on the definition of the DMR stability [23] and on the patients' management [30, 31]. In the present study, the achievement of an early DMR according to dPCR is associated with DMR stability, monitored either by RT-qPCR ($p = 0.0012$) or dPCR ($p = 0.0017$). These results imply that, in patients in the dPCR *pre* RT-qPCR group, both molecular approaches endorse DMR stability, indicating dPCR's improved potential for establishing DMR achievement. This suggests that dPCR could help the early selection of patients presenting a true DMR and who may attempt TFR before what estimated by RT-qPCR monitoring. Cases in the dPCR *pre* RT-qPCR group (44/79, 56%), the median time to DMR acquisition was 10 (3–39) and 18 (7–50) months for dPCR and RT-qPCR, respectively, with a median of the differences of 5 months. In contrast, in the dPCR *post* RT-qPCR group (10/79, 13%), the median time to DMR acquisition was 36 (13–68) and 25 (6–62) months for dPCR and RT-qPCR, respectively. This result suggests that there is a partial concordance between the two molecular techniques. This quantification resulted not influenced by the transcript type. In fact, in contrast with what recently observed in 2 prospective cohorts of 66 [32] and 67 patients [33], we did not find correlation between the transcript type neither when focusing on the dPCR DMR achievement time, nor when analysing the stability of the DMR by dPCR or RT-qPCR. It is important to note that transcript type distribution in our cohort is in line with what is reported by the literature, with a higher incidence of b3a2 (53/79, 67%) [11, 34]. Finally, in our experience the TKI type does not influence the time of achievement of the DMR and its stability according to dPCR. In terms of DMR achievement by dPCR, this aspect has been never investigated. Nevertheless, the same parameters have been shown to be influenced by TKI in case of monitoring via RT-qPCR [35, 36]. These differences can be attributed to the lower sensitivity and inherent limitations of RT-qPCR. This highlights that the achievement of a deep molecular response is influenced more significantly by the intrinsic characteristics of the disease rather than the efficacy of the TKIs. Altogether, these results appear to be of pivotal importance, as the transcript type and the TKI used, combined with time to achieve DMR and its stability, have been reported as influencing the TFR [37]. A new tool able to better assess the achievement of the DMR and its stability, such as dPCR, would contribute to the early identification of patients eligible for TKIs suspension for TFR purposes, as even patients lacking all conventionally defined optimal criteria for treatment discontinuation still may have good chance of TFR success [38].

Conclusions

In summary, this is the first study aiming at evaluating the effectiveness of dPCR in timely detecting DMR achievement and monitoring its stability in comparison with conventional RT-qPCR, and the impact of *BCR::ABL1* transcript type not only on the slope of transcript reduction but also on DMR stability. Our results confirmed that dPCR is probably the most useful tool for CML patients monitoring, and should be applied even from the first phases, after the start of TKIs therapy. Further studies, together with the standardization of the *BCR::ABL1* transcript quantification by dPCR are needed [39].

Abbreviations *CML*: Chronic Myeloid Leukemia; *TKI*: Tyrosine Kinase Inhibitor; *RT-qPCR*: Reverse transcription quantitative PCR; *dPCR*: Digital PCR; *MRD*: Minimal Residual Disease; *DMR*: Deep Molecular Response; *TFR*: Treatment-free remission; *MR*: Molecular response; *IS*: International scale

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Data availability Data are available at corresponding author upon request.

Declarations

Ethics approval The protocol was approved by the ethical committee of Verona, Milano, Udine, Brescia and Padua in July 2019.

Patient consent The studies were conducted in accordance with the Declaration of Helsinki and the enrolled patients gave their written informed consent.

Competing interests The authors declare no competing interests.

Clinical trial registration NP 3809 clinical trial.

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